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| (54) Title: BCL-2-ASSOCIATED PROTEINS | | |
| (57) Abstract <p>The present invention provides nucleotide sequences encoding proteins and fragments thereof that bind to Bcl-2-related proteins. The invention also provides a Bcl-2-associated protein (BAP) such as the BAG-1, which is encoded by the Bcl-2-associated athanogene-1 and binds to Bcl-2. The invention also provides antibodies that specifically bind to a BAP. The invention further provides methods for detecting agents such as drugs that alter the binding of a BAP such as BAG-1 or Raf-related protein with a Bcl-2-related protein and methods for detecting agents that induce dissociation of a bound complex formed by the association of a BAP and a Bcl-2-related protein. The invention further provides methods for modulating the activity of a Bcl-2-related protein in a cell by introducing into the cell a nucleic acid encoding a BAP or by introducing into the cell an antisense nucleotide sequence, which is complementary to a region of a gene encoding a BAP.</p> <div style="text-align: center; margin-top: 20px;"> <p>Handwritten notes:</p> <p>568,491 ← DIV → 08/152,485 → Pat → DIV → 5,641,866</p> <p> ↓ CIP</p> <p> 5,686,155 08/336,867 abd</p> </div> | | |

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BCL-2-ASSOCIATED PROTEINS

This work was supported by grant CA 47956 awarded by the National Cancer Institute of the National Institutes of Health. The United States Government has certain rights
5 in this invention.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates generally to the fields of molecular biology and molecular medicine and more
10 specifically to a novel protein that can regulate apoptosis.

BACKGROUND INFORMATION

The human body contains various tissues that continually undergo a process of self-renewal, whereby
15 older cells in the tissue die and are replaced by new cells. In order to maintain a relatively constant number of cells in a particular tissue, it is important that the number of dying cells in a tissue is equivalent to the number of newly produced cells. This homeostasis is
20 maintained by committing the cells in self-renewing tissues to a process of programmed cell death. The morphological features of cells undergoing this form of cell death has been termed "apoptosis." Though not all programmed cell deaths occur through apoptosis, as used herein, the terms
25 "programmed cell death" and "apoptosis" are used interchangeably.

Defects in the process of apoptosis occur in various pathological conditions. For example, in cancer, a defect in apoptosis results in cancer cells surviving
30 longer than their normal cell counterparts. As a result, the increased number of surviving cancer cells can cause an increase in the mass of a tumor, even if the doubling time

of the cancer cells is not increased above normal. In viral infections, induction of apoptosis can figure prominently in the pathophysiology of the disease process.

A protein, termed Bcl-2, plays a central role in the process of programmed cell death by blocking apoptosis. For example, when Bcl-2 levels in a cell are elevated, apoptosis is blocked. Conversely, when Bcl-2 levels in a cell are lowered, the rate of cell death is accelerated. Bcl-2 likely exerts its apoptosis-blocking effect at a final common pathway leading to apoptotic cell death. However, the molecular mechanism by which Bcl-2 blocks apoptosis remains enigmatic. An understanding of the role of Bcl-2 in the process of apoptosis is complicated by the observation that Bcl-2 is undetectable in some cell types that undergo apoptosis.

The presence of Bcl-2 in a cell also renders the cell highly resistant to various chemical and physical agents. In particular, Bcl-2 renders cancer cells more resistant to chemotherapeutic agents. Recently, various cellular and viral gene products have been identified that share a similar structure or activity with Bcl-2. Despite the similarity of Bcl-2 to these Bcl-2-related proteins, however, no structural feature of the Bcl-2 protein has been identified that lends a clue to its mechanism of regulating apoptosis.

One possible way Bcl-2 may act in the process of apoptosis is by regulating the activity of another protein involved in apoptosis. If so, the identification of an interaction between such a protein and Bcl-2 would allow the exploitation of this interaction in an assay to screen for agents such as drugs that alter the interaction. Such an assay would allow the identification of drugs that can regulate apoptosis and are useful for treating diseases such as neurodegenerative diseases or cancer or for

modifying the effectiveness of currently available cancer chemotherapeutic agents. Thus, a need exists to identify proteins that associate with Bcl-2 or Bcl-2-related proteins in order to modulate the regulation of apoptosis.

- 5 The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention relates to a nucleic acid molecule encoding a protein or a fragment thereof that
10 associates with Bcl-2 or a Bcl-2-related protein. The invention provides a Bcl-2-associated proteins (BAP) such as BAG-1, which is encoded by the Bcl-2-associated athanogene-1 and binds to Bcl-2. In addition, the invention relates to antibodies specific for a BAP.

- 15 The invention also relates to methods for detecting agents such as drugs that modulate the binding of a BAP with Bcl-2 or a Bcl-2-related protein and to methods for detecting agents that induce the dissociation of a bound complex formed by the association of a BAP and a Bcl-
20 2-related protein. For example, the invention provides solution and filter binding assays and the yeast two hybrid system assay, which are useful for detecting agents that increase or decrease the binding of a BAP or fragment thereof to a Bcl-2-related protein or that induce the
25 dissociation of a BAP and a Bcl-2-related protein.

- The invention further relates to methods for modulating the activity of Bcl-2 or a Bcl-2-related protein in a cell by introducing into the cell a nucleic acid encoding a BAP or by introducing into a cell an antisense
30 nucleotide sequence, which is complementary to a region of a gene encoding a BAP and can hybridize to a BAP gene in a cell or to an mRNA transcribed from the gene. Thus, the

invention provides methods for increasing or decreasing the ability of a cell to survive.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (SEQ ID NO: 1) shows the cDNA sequence
5 encoding the mouse Bcl-2-associated protein, BAG-1.

Figure 2 (SEQ ID NO: 2) shows the 219 amino acid sequence for the mouse BAG-1 protein, as deduced from the open reading frame present in the bag-1 cDNA sequence shown in Figure 1.

10 Figure 3 (SEQ ID NO: 3) shows a cDNA sequence encoding a portion of the human BAG-1 protein.

Figure 4 (SEQ ID NO: 4) shows a 189 amino acid portion of the human BAG-1 protein, as deduced from the open reading frame present in the bag-1 cDNA sequence shown
15 in Figure 3.

Figure 5 illustrates the amino acid sequence identity shared among mouse BAG-1 (mS33ORF.pep) and human BAG-1 (hs33.pep).

Figure 6 shows the migration of Bcl-2, which was
20 isolated by virtue of its association with BAG-1.

Figure 7 demonstrates that BAG-1 binds to an amino acid sequence present the N-terminal 218 amino acids of Bcl-2 in the yeast two-hybrid system.

Figures 8.A. to 8.C. show that gene transfer-
25 mediated elevations in BAG-1 and Bcl-2 in Jurkat T cell acute lymphocytic leukemia cells protect the Jurkat cells from cell death. All results represent mean +/- standard deviation (SD; n = 3).

In Figures 8.A. and 8.B., results are shown as percent cell viability at various times after exposure to 1 μ g/ml anti-Fas antibody 2D1 (Figure 8.A.; Fas) or to 10 μ M staurosporine (Figure 8.B.). Jurkat cells were transfected to express BAG-1 and/or Bcl-2 as indicated. "C" indicates cells transfected with a control plasmid. Cell viability was determined by an MTT dye binding assay.

In Figure 8.C., results are shown as percent Jurkat cell lysis 4 hr after addition of cytotoxic T lymphocytes (CTL), which had been transfected with a control plasmid (Neo) or to express a mutant activated Lck kinase (A-LCK) or a normal Lck kinase (N-LCK). Samples are as indicated. Cell lysis was determined by a chromium-51 release assay.

Figures 9.A. to 9.D. show the expression and function of gene transfer-mediated elevation of BAG-1 and Bcl-2 in cloned Jurkat cell lines.

Figure 9.A. and 9.B. show the expression of BAG-1 (Figure 9.A.) or Bcl-2 (Figure 9.B.) in Jurkat cells transfected with control plasmids (C) and in cloned cell lines obtained from Jurkat cells either transfected with a plasmid expressing Bcl-2 (7-2) or doubly transfected with plasmids expressing BAG-1 and Bcl-2 (5-2). Proteins were identified by immunoblot analysis. Arrows indicate the migration of BAG-1 (Figure 9.A.) and Bcl-2 (Figure 9.B.).

Figures 9.C. and 9.D. show the survival of the cloned cell lines and the control Jurkat cells 1 day after exposure to 1 μ g/ml anti-Fas antibody 2D1 (Figure 9.C.) or 5 days after exposure to 10 mM buthionine sulfoximine (BSO; Figure 9.D.). Data represent percent viable cells (mean \pm SD; n = 3) as determined by an MTT dye binding assay.

cells that were transfected with a plasmid that expresses
15 Bcl-2 (Bcl-2) or with a control plasmid (Neo).

Figure 11 presents a schematic diagram of a
method for screening drugs that can alter the association
of a BAP and a Bcl-2-related protein.

DETAILED DESCRIPTION OF THE INVENTION

20 The present invention provides nucleic acid
molecules encoding proteins or fragments thereof that bind
to Bcl-2 or to Bcl-2-related proteins. The invention
provides Bcl-2-associated proteins (BAP) such as the BAG-1
protein, which is encoded by the Bcl-2-associated
25 athanogene-1 and binds to Bcl-2. The Bcl-2-associated
athanogene-1 is named from the Greek word athanos, which
refers to anti-cell death. BAG-1 previously has been
referred to as Bcl-2-associated protein-1 or BAP-1.

Bcl-2 prolongs cell survival by blocking
30 apoptosis. In addition to maintaining homeostasis in self-
renewing tissues, Bcl-2 likely is involved in immune cell
"education" and the elimination of redundant neurons and

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other cell types during development. Bcl-2, which is an acronym for the protein encoded by the B cell lymphoma/leukemia-2 gene, was identified by its involvement in the t(14:18) translocation that is characteristic of a form of B cell lymphoma. Gene transfer experiments have demonstrated that Bcl-2 has oncogenic potential in a cell. As used herein, the term "Bcl-2" refers to the protein and the term "bcl-2" refers to the gene encoding Bcl-2. In cancer cells, Bcl-2 expression can result in increased cell survival, which contributes to the tumor mass.

Bcl-2 action occurs even in the complete absence of cell proliferation. Overexpression of Bcl-2 in a cell reduces the requirement of "competence" factors such as platelet derived growth factor and epidermal growth factor for growth of the cell in culture. Overexpression of Bcl-2 does not, however, affect the dependence of cells in culture on "progression" factors such as insulin-like growth factor-1. The role of Bcl-2 in apoptosis has been confirmed by showing that cells in which Bcl-2 levels are decreased due to expression of an antisense bcl-2 nucleic acid sequence have an accelerated rate of cell death. However, decreased Bcl-2 levels, alone, are not sufficient to cause cell death in many types of cells.

The present invention provides a nucleic acid molecule that encodes a BAP, which can associate with Bcl-2 or a Bcl-2-related protein and, thus, can be involved in the regulation of apoptosis. The invention provides, for example, a cDNA encoding mouse BAG-1 (Figure 1; SEQ ID NO: 1). The mouse bag-1 cDNA was cloned from a bacteriophage lambda EXLox expression vector library, which was screened using a recombinant Bcl-2 protein and an antibody to Bcl-2 (see Example I.A.).

Essentially, mouse cDNA was cloned into the lambda EXLox bacteriophage and the cDNA was expressed in *E.*

coli. The resultant plaques were screened using a recombinant Bcl-2 protein and binding of Bcl-2 was identified using an anti-Bcl-2 antibody, which was prepared as described by Reed et al., Anal. Biochem. 205:70-76 (1992), which is incorporated herein by reference. Positive phage were isolated and the cDNA inserts were sequenced. An 833 base pair cDNA was obtained and used to screen two other mouse cDNA libraries. The mouse bag-1 cDNA sequence shown in Figure 1 was obtained using this cloning strategy.

Although the lambda EXLox expression system was used to obtain the described mouse bag-1 cDNA, other expression vector systems such as lambda gt11 can be used to clone and express a cDNA such as the mouse bag-1 cDNA (see, for example, Sambrook et al., Molecular Cloning: A laboratory manual (Cold Spring Harbor Laboratory Press (1989), which is incorporated herein by reference). In addition, various other cloning and expression vectors are well known in the art and can be purchased from commercial sources.

Nucleic acid probes based on the mouse bag-1 cDNA sequence of Figure 1 can be used to screen a cDNA or genomic DNA library to obtain a nucleic acid encoding mouse bag-1 or a bag-1 cDNA from another species such as human. Such probes were used, for example, to obtain a cDNA sequence encoding human BAG-1 (see Example I.D.). Oligonucleotides corresponding to a portion of the nucleotide sequence present in Figure 1 can be synthesized using routine methods and used as hybridization probes to screen a library for a nucleic acid molecule having homology with the cDNA sequence encoding mouse BAG-1 (see Sambrook et al., *supra*, Chap. 11 (1989)). Oligonucleotides having a specific nucleotide sequence, including degenerate nucleotide sequences as desired, also can be purchased from commercial sources.

One skilled in the art would know that a screening method utilizing such oligonucleotides requires that hybridization be performed under relatively stringent conditions such that hybridization is relatively specific.

- 5 Appropriate hybridization conditions can be determined empirically or can be estimated based, for example, on the relative GC content of a sequence and the number of mismatches between the probe and the target sequence.

- Oligonucleotide probes also can be used to
- 10 identify genetic defects due a mutation of a gene encoding a BAP such as BAG-1 in a cell. Such a genetic defect can lead to aberrant expression of the BAP in the cell or to expression of an aberrant BAP, which does not properly associate with a Bcl-2-related protein in the cell. As a
- 15 result, a genetic defect in a gene encoding a BAP such as BAG-1 can result in a pathology characterized by increased or decreased apoptosis. As described above, the skilled artisan readily can determine relatively stringent hybridization conditions to identify, for example, a bag-1
- 20 gene containing a point mutation (see, for example, Sambrook et al., *supra*, 1989).

- Oligonucleotide probes as disclosed herein can be used to identify cells having a mutation in a gene encoding a BAP such as BAG-1 using well known hybridization methods.
- 25 In order to provide the specificity necessary to identify, for example, a point mutation in a BAG-1 gene, one skilled in the art would know that an oligonucleotide probe should be at least about fourteen to sixteen nucleotides in length (Sambrook et al., *supra*, 1989)). In addition, the probe
- 30 can incorporate a detectable moiety such as a radiolabel, a fluorochrome or a detectable binding agent such as biotin. These and other detectable moieties and methods of incorporating the moiety into an oligonucleotide probe are well known in the art and are commercially available.
- 35 However, a probe also can be useful, for example, as a

primer for the polymerase chain reaction (PCR). Such a probe useful as a primer can be unlabelled or labelled with a detectable moiety as desired.

The invention also provides a cDNA encoding a
5 portion of human BAG-1 (Figure 3; SEQ ID NO: 3). The human bag-1 cDNA was obtained using the mouse bag-1 cDNA as a hybridization probe to screen a human cDNA library (see Example I.D.). Positive clones were sequenced and the cDNA sequence shown in Figure 3 was obtained.

10 In addition, the human bag-1 cDNA sequence was used as a hybridization probe to screen a human cosmid library using methods as described below or otherwise known in the art (see, for example, Sambrook et al., *supra*, (1989)). Two positive cosmid clones were isolated and the
15 DNA inserts from the cosmids were used as probes for *in situ* hybridization to determine the chromosomal location of the human bag-1 gene. Using this method, the human bag-1 gene was localized to chromosome 9 at approximately band p13.

20 Since the amino acid sequences of the mouse and human BAG-1 proteins can be determined from the cloned nucleotide sequences shown in Figures 1 and 3, one skilled in the art can readily determine equivalent nucleotide sequences that also encode mouse and human BAG-1. As used
25 herein, an "equivalent" nucleotide sequence means a sequence that is different from that shown in Figure 1 or 3, but which encodes a protein having the identical amino acid sequence as shown in Figure 2 or 4, respectively. An equivalent nucleotide sequence is readily constructed by
30 incorporating silent nucleotide changes into a nucleotide sequence of Figure 1 or 3. Silent nucleotide changes are well known in the art and result from the degeneracy of the genetic code. One skilled in the art would readily recognize that a nucleotide sequence that is equivalent to

a sequence as shown in Figure 1 or 3 would encode a polypeptide as shown in Figure 2 or 4, respectively.

The invention also provides mouse BAG-1 and human BAG-1 proteins. The amino acid sequences of these proteins
5 were deduced from the nucleic acid sequences shown in Figures 1 and 3, respectively. The 219 amino acid mouse BAG-1 is shown in Figure 2 (SEQ ID NO: 2) and a 189 amino acid portion of the human BAG-1 protein is shown in Figure 4 (SEQ ID NO: 4). A comparison of the amino acid sequences
10 for mouse and human BAG-1 reveals that the two polypeptides share extensive sequence identity (Figure 5).

As used herein, the term "polypeptide" is used in its broadest sense to include proteins, polypeptides and peptides, which are related in that each consists of a
15 sequence of amino acids joined by peptide bonds. For convenience, the terms "polypeptide" and "protein" are used interchangeably. While no specific attempt is made to distinguish the size limitations of a protein and a peptide, one skilled in the art would understand that
20 proteins generally consist of at least about 50 to 100 amino acids and that peptides generally consist of at least two amino acids up to a few dozen amino acids. The term polypeptide as used herein includes any such amino acid sequence.

25 The invention also provides active fragments of BAG-1. As used herein, an "active fragment" of BAG-1 is a polypeptide that consists of an amino acid sequence derived from a BAP protein as shown, for example, in Figures 2 and 4 and that has the ability to associate with a Bcl-2-
30 related protein. An active fragment can be, for example, an N-terminal or C-terminal truncated form of a mouse or human BAG-1 protein (SEQ ID NOS: 2 and 4). Such truncated proteins can be produced using well known recombinant DNA methods.

Active fragments of a BAP, which can bind to a Bcl-2-related protein, are readily identified using the methods described in Examples II and V to VII, below). A particularly useful method for obtaining active fragments of BAG-1 is to create peptide phage display libraries as described, for example, in United States Patent No. 5,223,409, which is incorporated herein by reference, and screening such libraries with a Bcl-2-related protein as described in Example I. The usefulness of a peptide phage display library can be increased by using codon based mutagenesis to produce a diverse population of peptides as described by Huse (U.S. Patent No. 5,264,563, which is incorporated herein by reference).

A BAP is defined primarily by its ability to associate *in vitro* and *in vivo* with Bcl-2-related proteins. As used herein, the term "associate" means that a BAP and a Bcl-2-related protein have a binding affinity for each other such that the BAP and the Bcl-2-related protein form a bound complex. For convenience, the terms "associate" and "bind" are used interchangeably. The affinity of binding of a BAP and a Bcl-2-related protein is sufficiently specific such that the bound complex can form *in vivo* in a cell and can form *in vitro* under appropriate conditions as described herein. The formation or dissociation of a bound complex can be identified as described in Examples II and VIII or using other well known methods such as equilibrium dialysis.

As described herein, mouse BAG-1 and human BAG-1 are examples of Bcl-2-associated proteins. Other examples of BAP's include the Raf-related proteins such as A-Raf, B-Raf or the Raf-1 kinase, which is a serine/threonine-specific kinase having a molecular mass of about 72 to 74 kiloDaltons (kDa). The Raf-1 kinase, for example, has an amino-terminal regulatory domain and a carboxyl-terminal catalytic domain. Removal of the regulatory domain by

deletion mutagenesis releases the catalytic domain from suppression and produces a kinase having a constitutively high level of enzymatic activity (Heidecker et al., Mol. Cell. Biol. 10:2503-2512 (1990), which is incorporated
5 herein by reference). In some cell types and under some conditions, the presence of Bcl-2 alone is not sufficient to block apoptosis. However, whereas neither Bcl-2 nor Raf-1 alone can suppress apoptosis, the combination of Bcl-2 and the Raf-1 kinase suppresses apoptosis. As described
10 in Example VI, below, Raf-1 associates with Bcl-2 in vivo and, therefore, has the characteristics of a Bcl-2-associated protein.

As used herein, the term "Bcl-2-related" proteins refers to proteins that are structurally or functionally
15 related to Bcl-2. For convenience, the term "Bcl-2-related proteins" is used generally to include Bcl-2, itself. Bcl-2-related proteins can be identified by their ability to associate with a BAP such as BAG-1 under appropriate conditions (see Examples II, III and V). Several genes
20 encoding Bcl-2-related proteins are known. For example, the bcl-2 gene is highly homologous to bcl-X, which encodes two proteins, Bcl-X-L and Bcl-X-S, formed via alternative splicing. Bcl-2 also has a weak homology with an open reading frame, BHRF-1, in Epstein Barr Virus (Cleary et al., Cell 47:19-28 (1986), which is incorporated herein by
25 reference) and with the *C. elegans* ced-9 gene, which, when overexpressed, prevents programmed cell deaths during worm development (Hengartner et al., Nature 356:494-499 (1992); Vaux et al., Science 258:1955-1957 (1992), each of which is
30 incorporated herein by reference). In addition, the mcl-1 gene and the A1 gene, which have moderate homology with bcl-2 and BHRF-1, are Bcl-2-related proteins that have been identified in leukemic and normal myeloid cells, respectively, that were induced to differentiate using
35 phorbol ester or colony stimulating factors (see, for example, Lin et al., J. Immunol. 151:1979-1988 (1993),

which is incorporated herein by reference). Although the function of these Bcl-2-related genes has not been described, the mcl-1 and Al gene products may have roles in apoptosis in cells. Bcl-2 β , which is an alternatively
5 spliced form of Bcl-2, is another example of a Bcl-2-related protein as is Bax (Oltvai et al., Cell 74:609-619 (1993), which is incorporated herein by reference).

All Bcl-2-related proteins identified thus far contain significant amino acid similarity in at least one
10 of three well conserved domains, which have been designated domain A (residues 11 to 28 of human Bcl-2), domain B (residues 140 to 149) and domain C (residues 188 to 196) (Sato et al., Gene 140:291-292 (1994), which is incorporated herein by reference). BAG-1 lacks similarity
15 to these three conserved domains and, therefore, is not a member of the Bcl-2-related protein family.

In view of the nucleic acid sequences encoding BAG-1 and the BAG-1 amino acid sequences that are provided herein, one skilled in the art would know how to synthesize
20 specific BAG-1 peptides or an entire BAG-1 protein, if desired, using routine methods of solid phase peptide synthesis (see Example IV). In addition, analogs of BAG-1 or BAG-1 peptides can be designed to have increased stability *in vivo* or *in vitro* or higher or lower affinity
25 of binding to a Bcl-2-related protein by incorporating, for example, (D)-amino acids into a BAG-1 peptide or by chemically modifying reactive amino acid side chains or the amino or carboxy terminus of a peptide. For example, a reactive amino group in a peptide can be rendered less
30 reactive by acetylation. Furthermore, a modification such as acetylation changes a hydrophilic group to a hydrophobic group, which can be advantageous, for example, when it is desirable to prepare a BAG-1 peptide that can readily traverse a cell membrane.

The invention further provides antibodies specific for BAG-1. As used herein, the term "antibody" is used in its broadest sense to include polyclonal and monoclonal antibodies, as well as polypeptide fragments of antibodies that retain a specific binding activity for a BAP of at least about $1 \times 10^5 \text{ M}^{-1}$. One skilled in the art would know that anti-BAG-1 antibody fragments such as Fab, F(ab')₂, and Fv fragments can retain specific binding activity for BAG-1 and, thus, are included within the definition of an antibody. In addition, the term "antibody" as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies and fragments that retain binding activity such as chimeric antibodies or humanized antibodies. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as described by Huse et al., Science 246:1275-1281 (1989), which is incorporated herein by reference.

As described in Example IV, below, anti-BAG-1 antibodies were prepared using a BAG-1 fusion protein or a synthetic peptide encoding a portion of mouse BAG-1 as immunogen. One skilled in the art would know that purified BAG-1, which can be prepared from natural sources or produced recombinantly, or fragments of BAG-1, including a peptide portion of BAG-1 such as a synthetic peptide as described above, can be used as an immunogen. Non-immunogenic fragments or synthetic peptides of BAG-1 can be made immunogenic by coupling the hapten to a carrier molecule such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), as described in Example IV, below. In addition, various other carrier molecules and methods for coupling a hapten to a carrier molecule are well known in the art and described, for example, by Harlow and Lane, Antibodies: A laboratory manual (Cold Spring Harbor

Laboratory Press, 1988), which is incorporated herein by reference.

Since BAG-1 and Bcl-2, for example, associate in a cell in vivo (see Example V), a pathology that is characterized by an increased or decreased level of apoptosis, as compared to the level of apoptosis known to occur in a healthy tissue, may be due to an increased or decreased level of BAG-1 in a cell. Thus, it can be desirable to determine whether BAG-1 levels in a cell are within the normal range expected for a particular cell type in a tissue or organ. Anti-BAG-1 antibodies are useful for determining the level of BAG-1 in a tissue sample, which can be diagnostic of a pathology. Such a determination can be made using immunoassay and immunohistochemical methods as described in Examples IV and V or otherwise known in the art (see, for example, Reed et al. (1992); see, also, Harlow and Lane (1988)).

A diagnostic kit incorporating an anti-BAG-1 antibody as an assay for detecting a pathology due to altered BAG-1 expression in a cell is particularly useful. Such a kit can contain, in addition to an anti-BAG-1 antibody, a reaction cocktail that provides the proper conditions for performing the assay, control samples that contain known amounts of BAG-1 and, if desired, a second antibody specific for the anti-BAG-1 antibody. A diagnostic assay should include a simple method for detecting the amount of BAG-1 in a sample that is bound to the antibody. Detection can be performed by labelling an anti-BAG-1 antibody using methods well known in the art (see, for example, Harlow and Lane, 1988; chap. 9). For example, an antibody can be labelled with various detectable moieties including a radiolabel, an enzyme, biotin or a fluorochrome. Reagents for labelling an anti-BAG-1 antibody can be included in the diagnostic kit or can be purchased separately from a commercial source.

Following contact of a labelled antibody with a sample such as a tissue homogenate or a histological section of a tissue, specifically bound labelled antibody can be identified by detecting the particular moiety.

5 Alternatively, a labelled second antibody can be used to identify specific binding of an unlabelled anti-BAG-1 antibody. A second antibody generally will be specific for the particular class of the first antibody. For example, if an anti-BAG-1 antibody is of the IgG class,
10 a second antibody will be an anti-IgG antibody. Such second antibodies are readily available from commercial sources. The second antibody can be labelled using a detectable moiety as described above. When a sample is labelled using a second antibody, the sample is first
15 contacted with a first antibody, then the sample is contacted with the labelled second antibody, which specifically binds to the first antibody and results in a labelled sample.

Example IV provides a detailed method for raising
20 polyclonal anti-BAG-1 antibodies in rabbits. In addition, monoclonal antibodies can be obtained using methods that are well known and routine in the art (Reed et al. (1992); Harlow and Lane (1988)). Essentially, spleen cells from a BAG-1-immunized mouse can be fused to an appropriate
25 myeloma cell line such as SP/02 myeloma cells to produce hybridoma cells. Cloned hybridoma cell lines can be screened using labelled BAG-1 protein to identify clones that secrete anti-BAG-1 monoclonal antibodies and hybridomas that express antibodies having a desirable
30 specificity and affinity can be isolated and utilized as a continuous source of anti-BAG-1 antibodies. One skilled in the art would know that a dependable source of monoclonal anti-BAG-1 antibodies is desirable, for example, for preparing diagnostic kits as described above.

The invention further provides methods for detecting agents such as drugs that can modulate the ability of a BAP to associate with a Bcl-2-related protein and methods for detecting agents that induce dissociation
5 of a bound complex formed by the association of a BAP and a Bcl-2-related protein. As used herein, the term "modulate" is used in its broadest sense to mean increase or decrease. Examples of screening assays for detecting agents that can modulate the association of a BAP and a
10 Bcl-2-related protein or induce the dissociation of such a BAP and a Bcl-2-related protein are described in Example VIII (see, also, Figure 11).

As used herein, the term "agent" means a chemical or biological molecule such as a simple or complex organic
15 molecule, a peptide, a protein or an oligonucleotide that is useful as a drug. In addition, the term "effective agent" is used herein to mean an agent that, in fact, is useful as a drug. The screening assays described herein are particularly useful in that they can be automated,
20 which allows for high through-put screening of randomly designed agents to identify useful drugs, which can modulate the ability of a BAP and a Bcl-2-related protein to associate. For example, a drug can alter the ability of a BAP and a Bcl-2-related protein to associate by
25 decreasing or inhibiting the binding affinity of a BAP and a Bcl-2-related protein.

A drug screening assay can utilize BAG-1 or, as exemplified in Figure 11, a BAG-1 fusion protein such as a BAG-1-glutathione-S-transferase (GST) or BAG-1-histidine-6
30 fusion protein (see Examples II.A. and VIII). The BAG-1 or BAG-1 fusion protein is characterized, in part, by having an affinity for a solid substrate as well as having an affinity for a Bcl-2-related protein. For example, when BAG-1 is used in the assay, the solid substrate can contain
35 a covalently attached anti-BAG-1 antibody. Alternatively,

a BAG-1-GST fusion protein can be used in the assay. Where such a fusion protein is used in the assay, the solid substrate can contain covalently attached glutathione, which is bound by the GST component of the BAG-1-GST fusion
5 protein.

The drug screening assay can be performed by allowing the BAP or BAP-fusion protein to bind to the solid support, then adding a Bcl-2-related protein and a drug to be tested (see Example VIII). Control reactions will not
10 contain the drug (see Examples II and III). Following incubation of the reaction mixture under conditions known to be favorable for the association, for example, of BAG-1 and Bcl-2 in the absence of a drug, the amount of Bcl-2 specifically bound to BAG-1 in the presence of a drug can
15 be determined.

An effective agent can be useful as a drug for modulating the association of a BAP and a Bcl-2-related protein. For example, a drug that decreases or inhibits the association of a BAP and a Bcl-2-related protein can be
20 useful where it is desirable to increase the concentration of unbound Bcl-2 in a cell, for example, so that free Bcl-2 is available to block apoptosis. Alternatively, a drug can be useful for increasing the affinity of binding of a BAP and a Bcl-2-related protein.

For ease of detecting binding, the Bcl-2 protein
25 can be labelled with a detectable moiety, such as a radionuclide or a fluorescent label, as described above and in Example VIII, below. By comparing the amount of specific binding of BAG-1 and Bcl-2 in the presence of a
30 drug as compared to the control level of binding, a drug that increases or decreases the binding of a BAP and a Bcl-2-related protein can be identified. Thus, the drug screening assay provides a rapid and simple method for selecting agents having a desirable effect on the

association of a BAP and a Bcl-2-related protein. Such agents can be useful for modulating the activity of Bcl-2 or a Bcl-2-related protein in a cell and, therefore, can be useful as medicaments for the treatment of a pathology
5 caused by an altered level of apoptosis.

A transcription activation assay such as the yeast two hybrid system as described in Example II.C. also is useful as a screening assay to identify effective agents that alter interactions among a BAP and Bcl-2 or a Bcl-2-
10 related protein such as Bax. A transcription activation assay can be used to screen a panel of agents to identify an effective agent, which can be useful for increasing or decreasing apoptosis in a cell.

An effective agent can be identified by detecting
15 an altered level of transcription of a reporter gene (see Examples II.C. and VIII). For example, the level of transcription of a reporter gene due to the bridging of a DNA-binding domain and trans-activation domain by a BAP and Bcl-2 or a Bcl-2-related protein can be determined in the
20 absence and in the presence of an agent. An effective agent that increases the interaction between a BAP and Bcl-2 or a Bcl-2 related protein can be identified by an increased level of transcription of the reporter gene as compared to the control level of transcription in the
25 absence of the agent.

For example, the interaction can be the binding of a BAG-1 hybrid with a Bcl-2 hybrid. As described below, BAG-1 and Bcl-2 can bind and the expression of these proteins in a cell increases the resistance of the cell to
30 apoptosis (see Figure 7 to 9). Thus, the interaction of BAG-1 and Bcl-2, in part, can regulate the ability of a cell to survive. As used herein, the term "ability of a cell to survive" is used in a relative sense to indicate the life span of a cell exposed, for example, to a

cytotoxic agent, as compared to the normal life span of a cell. For example, exposure of a cell to a cytotoxic agent can decrease the life span of the particular cell and, therefore, the ability of the cell to survive. As
5 disclosed herein, the present invention provides methods and compositions that can increase or decrease the ability of such a cell to survive.

An agent that effectively increases the interaction of BAG-1 and Bcl-2, as detected by increased
10 transcription of the reporter gene in a two-hybrid assay, can decrease the level of apoptosis in a cell. Such an effective agent can be particularly useful as a medicament for treating a patient suffering from a disease characterized by a high level of apoptosis such as a
15 neurodegenerative disease. Such an agent also can be useful, for example, to prolong the time a cell such as a hybridoma cell can survive in culture and, therefore, can improve bioproduction yields in industrial tissue culture applications.

20 An effective agent that decreases the interaction of a BAP and a Bcl-2-related protein also can be identified, in this case by detecting a decreased level of transcription of a reporter gene as compared to the level of transcription in the absence of the agent. For example,
25 an agent that decreases the interaction of BAG-1 and Bcl-2 in a cell can increase the level of apoptosis in the cell. Such an effective agent can be useful, for example, to increase the level of apoptosis of a cancer cell, which is characterized by having a decreased level of apoptosis as
30 compared to its normal cell counterpart. Thus, effective agents identified using the methods described herein are particularly useful as medicaments to increase or decrease the level of apoptosis in a cell in a subject.

In some cases, an agent may not be able to cross the yeast cell wall and, therefore, cannot enter the yeast cell to alter an interaction among members of a BAP and Bcl-2 or a Bcl-2-related protein. The use of yeast spheroplasts, which are yeast cells that lack a cell wall, can circumvent this problem (Smith and Corcoran, In Current Protocols in Molecular Biology (ed. Ausubel et al.; Green Publ., NY 1989), which is incorporated herein by reference).

10 In addition, a potentially effective agent, upon entering a cell, may require "activation" by a cellular mechanism, which may not be present in yeast. Activation of an agent can include, for example, metabolic processing of the agent or a modification such as phosphorylation of
15 the agent, which can be necessary to convert the agent into an effective agent. In this case, a mammalian cell line can be used to screen a panel of agents. A transcription assay such as the yeast two-hybrid system described in Example II.C. can be adapted for use in mammalian cells using well known methods (Fearon et al., Proc. Natl. Acad. Sci., USA 89:7958-7962 (1992), which is incorporated herein by reference; see, also, Sambrook et al., Molecular Cloning: A laboratory manual (Cold Spring Harbor Laboratory Press 1989), and Ausubel et al., Current
20 Protocols in Molecular Biology (Green Publ., NY 1989), each of which is incorporated herein by reference).

The invention further provides methods for modulating the activity of Bcl-2 or a Bcl-2-related protein for preventing apoptosis in a cell by introducing into the
30 cell a nucleotide sequence encoding BAG-1 or an antisense nucleotide sequence, which is complementary to a region of a gene encoding BAG-1 and can hybridize to the gene or to an mRNA transcribed from the gene. As disclosed herein, modulation of the level of BAG-1 in a cell can increase or
35 decrease the ability of a cell to survive (see Example

VII). Thus, the nucleotide sequences described herein can be used as medicaments for the treatment of a pathology characterized by an altered level of apoptosis.

The level of a gene product such as BAG-1 can be increased in a cell using recombinant expression vectors and gene transfer technology to express a nucleic acid encoding BAG-1 or an active fragment of BAG-1. Increased expression of BAG-1, either alone or in combination with increased expression of Bcl-2, in a cell can increase the ability of the cell to survive following exposure to various cytotoxic agents (see Example VII and Figures 8 to 10).

Various expression vectors and methods for introducing such vectors into a cell are well known in the art and are described, for example, in Sambrook et al., *supra*, (1989). Viral vectors that are compatible with a targeted cell are particularly useful for introducing a nucleic acid encoding BAG-1 into a cell. For example, recombinant adenoviruses having general or tissue-specific promoters can be used to deliver BAG-1 expression constructs into a variety of types of tissues and cells, including non-mitotic cells, and to drive bag-1 cDNA expression in the target cells. Recombinant adeno-associated viruses also are useful and have the added advantage that the recombinant virus can stably integrate into the chromatin of even quiescent non-proliferating cells such as neurons of the central and peripheral nervous systems (Lebkowski et al., Mol. Cell. Biol. 8:3988-3996 (1988), which is incorporated herein by reference).

Such viral vectors are particularly useful where one skilled in the art desires to provide a nucleic acid encoding BAG-1 to a subject, for example, for gene therapy. Viruses are specialized infectious agents that have evolved in many cases to elude host defense mechanisms and to

infect and propagate in specific cell types. The targeting specificity of viral vectors can be utilized to target predetermined cell types and introduce a recombinant gene into the infected cell. Thus, the viral vector selected
5 will depend, in part, on the cell type to be targeted. For example, if neurodegenerative diseases are to be treated by increasing the BAG-1 levels in neuronal cells affected by a disease, then a vector specific for cells of the neuronal cell lineage can be used. Such viral vectors include, for
10 example, Herpes simplex virus-based vectors (Battelman et al., J. Neurosci. 13:941-951 (1993), which is incorporated herein by reference). Similarly, if a disease or pathological condition of the hematopoietic system is to be treated, then a viral vector that is specific for blood
15 cells and their precursors, preferably for the specific type of hematopoietic cell, can be used. Such viral vectors include, for example, HIV-based vectors (Carroll et al., J. Cell. Biochem. 17E:241 (1993), which is incorporated herein by reference).

20 Vectors such as those described herein also can express specific receptors or ligands, which can modify or alter target specificity through receptor mediated events. Such vectors can be constructed using recombinant DNA techniques or synthetic chemistry procedures. In addition,
25 a viral vector can be made tissue-specific by incorporating a tissue-specific promotor or enhancer into the vector (Dai et al., Proc. Natl. Acad. Sci. USA 89:10892-10895 (1992), which is incorporated herein by reference).

Retroviral vectors are often preferred for in
30 vivo targeting and therapy procedures. Retroviral vectors can be constructed either to function as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains the necessary genes,
35 regulatory sequences and packaging signals to synthesize

new viral proteins and RNA. However, genes conferring oncogenic potential of these viruses is destroyed. After the viral proteins are synthesized, the host cell packages the RNA into new viral particles, which can undergo further rounds of infection. The viral genome also is engineered to encode and express the desired recombinant gene.

In the case of non-infectious viral vectors, the helper virus genome is usually mutated to destroy the viral packaging signal required to encapsulate the RNA into viral particles. However, the helper virus retains structural genes required to package a co-introduced recombinant virus containing a gene of interest. Without a packaging signal, viral particles will not contain a genome and, thus, cannot proceed through subsequent rounds of infection.

Methods for constructing and using viral vectors are known in the art and are reviewed, for example, in Miller and Rosman, Biotechniques 7:980-990 (1992), which is incorporated herein by reference. The specific type of vector will depend upon the intended application. These vectors are well known and readily available within the art or can be constructed by one skilled in the art.

For gene therapy, BAG-1-encoding expression vectors can be administered to a subject in various ways to obtain increased levels of BAG-1 in the cells affected by a disease or pathological condition. For example, if viral vectors are used, the procedure can take advantage of their target cell specificity and the vectors need not be administered locally at the diseased site. However, local administration can provide a quicker, more effective treatment. Administration also can be by intravenous or subcutaneous injection into the subject. Injection of viral vectors into the spinal fluid can be used as a mode of administration, especially in the case of neurodegenerative diseases. Following injection, the viral

vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

Receptor-mediated DNA delivery approaches also can be used to deliver BAG-1 expression plasmids into cells in a tissue-specific fashion using a tissue-specific ligand or antibody non-covalently complexed with DNA via bridging molecules (Curiel et al., Hum. Gene Ther. 3:147-154 (1992); Wu and Wu, J. Biol. Chem. 262:4429-4432 (1987), each of which is incorporated herein by reference). Direct injection of DNA or DNA encapsulated in cationic liposomes also can be used for stable gene transfer to non-dividing and dividing cells *in vivo* (Ulmer et al., Science 259:1745-1748 (1993), which is incorporated herein by reference). In addition, DNA can be transferred into a variety of tissues using the particle bombardment method (Williams et al., Proc. Natl. Acad. Sci. USA 88:2726-2730 (1991), which is incorporated herein by reference).

A particularly useful mode of administration of BAG-1 encoding vectors is by direct inoculation locally at the site of the disease or pathological condition. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve BAG-1 expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement necessary with other forms of administration. Thus, a vector that infects all cells in the inoculated area can be used. If expression is desired in only a specific subset of cells within an inoculated area, then promotor and expression elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be viral vectors, viral genomes, plasmids, phagemids and the like. Transfection vehicles such as liposomes also can be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such

transfection vehicles are well known in the art. Transfection methods also are useful for introducing a BAG-1-containing vector into a cell in culture. Such methods, including calcium phosphate precipitation, DEAE-dextran
5 facilitated transfection and lipofection methods are well known in the art and reagents for performing these methods are commercially available (Sambrook et al., *supra*, 1989).

The level of BAG-1 in a cell also can be modulated such that BAG-1 levels in the cell are decreased.
10 As a consequence of decreased BAG-1 levels, free Bcl-2 levels in a cell can be increased. Various methods using, for example, ribozymes or homologous recombinant gene knock-out can be used to decrease the level of BAG-1 in a cell (see, for example, Capecchi, Nature 344:105 (1990) and
15 references cited therein; McCall et al., Proc. Natl. Acad. Sci., USA 89:5710-5714 (1992), each of which is incorporated herein by reference).

One method for decreasing the expression of a protein such as BAG-1 in a cell is to introduce into the
20 cell an antisense RNA-producing expression vector or synthetic antisense oligonucleotides, which are complementary to a nucleotide sequence of a BAG-1 gene (see, for example, Godson et al., J. Biol. Chem. 268:11946-11950 (1993); Reed et al., Canc. Res. 50:6565-6570 (1990a);
25 Reed et al., Proc. Natl. Acad. Sci., USA 87:3660-3664 (1990b), each of which is incorporated herein by reference). Antisense oligonucleotides can be purchased or can be synthesized as described above.

Antisense RNA can be produced in a cell using
30 expression vectors as described above. In this case, expression from the vector can result, for example, in the production of antisense RNA in a cell (see Reed et al., *supra*, (1990b)). Alternatively, synthetic antisense oligonucleotides can be introduced directly into cells or

can be encapsulated, for example, in liposomes, which facilitate transfer of oligonucleotides into a cell. Where antisense oligonucleotides are directly administered, it can be desirable to construct the oligonucleotides using
5 nucleotide analogs, which can confer increased stability on the oligonucleotide *in vivo*, as described, for example, by Reed et al., *supra*, (1990a)).

Expression of a BAP in a cell also can provide a therapeutic advantage in treating a subject such as a
10 cancer patient with a chemotherapeutic agent. Bcl-2 expression in a cell has been found to prevent or markedly reduce cell killing induced by a wide variety of physical and chemical agents including, for example, 1) calcium ionophores, phorbol esters, cAMP derivatives, anti-T cell
15 receptor complex antibodies and glucocorticoids in primary thymocytes and leukemic T cell lines; 2) gamma-irradiation in immature hematolymphoid cells; 3) heat shock; 4) glucose deprivation, free radicals, lipid peroxidation and glutamate in PC12 rat pheochromocytoma and other neuronal
20 cell lines; 5) some type of viruses including baculovirus in insect cells and Sindbis virus in mammalian cells; and 6) various chemotherapeutic drugs that act by different mechanisms, including vincristine and taxol, which inhibit microtubule formation, cytosine arabinoside, which is an
25 antimetabolite, methotrexate, which is an inhibitor of nucleotide synthesis, etoposide and mitozantron, which inhibit topoisomerase, adriamycin and daunomycin, which intercalate into DNA, cyclophosphamide congeners, which are alkylating agents, and BCNU (a nitrogen mustard), 2-
30 chlorodeoxyadenosine and cisplatin. As disclosed herein, expression of BAG-1 in a cell also can increase the ability of a cell to survive various cytotoxic insults (see Figures 8 to 10). Thus, expression of a BAP in a cell using the methods described above can decrease the susceptibility of
35 a cell to apoptosis induced by these or other chemical or physical agents. A therapeutic advantage can be attained,

for example, by increasing the expression of a BAP in normal cells, but not tumor cells, so that the ability of the normal cells to survive is increased.

The following examples are intended to illustrate
5 but not limit the present invention.

EXAMPLE I

Isolation and Characterization of the Mouse and Human bag-1 cDNA Sequences and BAG-1 Protein

This example describes methods for isolating and
10 characterizing the mouse and human bag-1 cDNA sequences and BAG-1 proteins.

A. Identification and characterization of a nucleic acid sequence encoding mouse BAG-1

A sandwich immunoassay was devised for detection
15 of novel Bcl-2-associated proteins produced from bacteriophage lambda-infected *E. coli*. The cDNA expression library used for this cloning procedure represented cDNA that had been derived from a day 11 mouse embryo and cloned into the lambda phage vector, EXlox (library purchased from
20 Novagen, Inc.). *E. coli* strain BL21(DE3)pLysE cells were infected with the bacteriophage lambda-EXlox library and the infected cells were suspended in semi-solid growth medium (0.72% agarose) in 100 mm or 150 mm petri dishes.

Cells were incubated at 37°C until plaques of 1-2
25 mm diameter formed. The plaque density was adjusted empirically to yield approximately 1×10^4 plaques per 100 mm dish or 5×10^4 plaques per 150 mm dish. Nitrocellulose circles were soaked in 10 mM IPTG, then dried and placed on the surface of the cultures. Incubation was continued at
30 37°C for 3 to 16 hrs to induce production of recombinant proteins from the lambda phages. The filters were removed from the dishes, dried to fix the proteins and pre-blocked

in a solution containing 75 mM KCl, 20 mM Hepes (pH 7.7), 2.5 mM MgCl₂, 2 mM EGTA, 1 mM PMSF, 0.05% Triton X-100, 1 mM dithiothreitol (DTT) and 5% non-fat dried milk powder.

Recombinant Bcl-2 protein was produced in Sf9
5 insect cells using recombinant bcl-2 DNA-containing baculoviruses as described by Reed et al., *supra*, (1992). Baculovirus-infected cells were suspended in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 8), 5mM EDTA and protease inhibitors (protease inhibitors
10 include 1 mM phenylmethylsulfonyl fluoride, 0.23 unit/ml aprotinin, 10 μ M leupeptin and 1 mM benzamidine). The resulting suspension contained approximately 200 ug/ml recombinant Bcl-2 protein.

The crude Bcl-2 protein preparation was added to
15 solution A (75 mM KCl, 20 mM Hepes (pH 7.7), 2.5 mM MgCl₂, 0.1 mM EGTA, 0.05% Triton X-100, 1 mM PMSF, 1mM DTT and 1% non-fat dried milk) to achieve a final Bcl-2 protein concentration of approximately 2 μ g/ml. Plaque-lifts were incubated overnight with the Bcl-2-containing solution at
20 4°C to allow Bcl-2 protein to bind to phage that expressed Bcl-2-binding protein fragments. Following binding, filters were washed 1x at room temperature in solution A, then incubated for 1-2 hrs with 0.1% v/v solution A and ascites fluid from the 4D7 hybridoma, which produces a
25 monoclonal antibody directed against amino acids 61-76 of the human Bcl-2 protein (Reed et al., *supra*, (1992)). The Bcl-2 antibody complexes were detected on the filters using alkaline phosphatase-conjugated rabbit anti-mouse IgG (Promega; Madison, WI). Color development was achieved
30 using nitroblue tetrazolium/bromochloroindoyl phosphate. One positive clone was detected after screening approximately 1 x 10⁶ plaques. The interaction of Bcl-2 with this positive phage clone was confirmed using an additional anti-Bcl-2 antibody, Mab100 (Dako, Inc.;

Carpinteria CA). Reactions using Mab100 were performed as recommended by the supplier.

The positive clone was plaque purified and the cDNA insert was transferred from lambda phage to plasmid using the loxP-cre system. Briefly, BM25.8 cells that contain the Pl-cre recombinase were infected with the lambda bacteriophage and grown in medium containing 50 µg/ml carbenicillin (Palazzolo et al., Gene 66:25-36 (1990); Margolis et al., Proc. Natl. Acad. Sci., USA 89:8894-8878 (1992), each of which is incorporated herein by reference). Plasmid DNA was purified from the resulting antibiotic-resistant colonies of bacteria and both strands were sequenced by the dideoxynucleotide termination method using T7 DNA polymerase. One plasmid, pEXlox-BAG-1, contained an 833 bp nucleic acid sequence, including 630 nucleotides encoding an open reading frame.

The 833 bp cDNA was isolated from pEXlox-BAG-1 by digestion with EcoRI and Hind III and labelled by a random primer method using α -³²P-dCTP for use as a hybridization probe to obtain overlapping cDNA sequences from a mouse spleen cell cDNA library and a mouse kidney cDNA library (Clontech, Inc.; Palo Alto CA). Plasmid pSN-245-9, which contains nucleotides -82 to +843 of the bag-1 cDNA sequence, was isolated. Parts of the 5'-end of the cDNA sequence were derived using the rapid enzymatic amplification of cDNA ends ("RACE") procedure described by Froehman et al., Proc. Natl. Acad. Sci., USA 85:8998-9002 (1988), which is incorporated herein by reference. The nucleotide sequence of mouse bag-1 cDNA is shown in Figure 1 (SEQ ID NO: 1).

30 B. Expression of RNA encoding BAG-1

RNA expression in various mouse tissues was examined by northern blot analysis. RNA was isolated from various tissues of 4 wk C57Bl/6 male mice using a

guanidinium-isothiocyanate method and CsCl centrifugation as described by Reed et al. (Mol. Cell. Biol. 5:3361 (1985), which is incorporated herein by reference). RNA was denatured using glyoxal, size-fractionated in 1% agarose gels, and transferred to nylon filters (Zeta Probe; Biorad) in 7.8 mM NaOH for 6 hr. The blots were washed 2x for 5 min each in 2X SSC/0.1% SDS (Sambrook et al., *supra*, 1989).

Prehybridization was performed in 1M NaCl, 1% SDS and 10% dextran-sulfate, containing 200 µg denatured salmon sperm DNA. Hybridization was performed in the same solution containing a ³²P-bag-1 probe. Washes were performed twice each using 2X SSC/0.1% SDS at 25 °C for 5 min., 2X SSC/0.1% SDS at 60 °C for 20 min, and 0.1X SSC/0.1% SDS at 25 °C for 20 min.

A 1.8 kilobase pair bag-1 transcript was detected in a wide variety of tissues (not shown). The highest relative levels of bag-1 RNA were present in testes. Lung, kidney, stomach, small intestine and colon also contained relatively high levels of bag-1 mRNA. In addition, bag-1 RNA was expressed in thymus, spleen, lymph nodes, skin, brain, muscle and bone marrow. However, little if any bag-1 RNA was detected in liver, which also does not express bcl-2 in the mouse (not shown). The blots also were hybridized with a probe for glyceraldehyde phosphate dehydrogenase (GAPDH) and stained to detect the 28s and 18s ribosomal RNA bands to confirm that equivalent amounts of intact RNA were loaded in each lanes.

C. Characterization of the mouse BAG-1 protein

The mouse bag-1 cDNA sequence contains an open reading frame encoding a putative 219 amino acid protein (Figure 2; SEQ ID NO: 2). The first potential start codon (AUG) for translation initiation within this open reading

frame conforms well to the usual Kozak consensus sequence (8-10 matches; Kozak, J. Biol. Chem. 266:19867-19870 (1991)). Two additional in-frame AUG sequences are located at positions +19 bp and +37 bp. However, the latter
5 sequences occur within less favorable sequence contexts for translation initiation (Kozak, *supra*, 1991).

Comparison of the nucleotide sequence and the deduced amino acid sequence of BAG-1 with sequences available in the Genbank, EMBL and Swiss-Protein databases
10 did not reveal any homologous sequences. Furthermore, BAG-1 is not homologous to Bcl-2 or the members of the Bcl-2-related family of proteins. However, the deduced mouse BAG-1 protein is approximately 85% homologous to human BAG-1 (see below; see, also, Figure 5).

15 The deduced mouse BAG-1 amino acid sequence is rich in glutamic acid residues (31 of 219 residues). The predicted isoelectric point of BAG-1 is pI 4.81, which indicates that BAG-1 is an acidic protein. Kyte-Doolittle plots did not reveal the presence of hydrophobic domains,
20 which are characteristic of a transmembrane domain or a hydrophobic leader sequence.

Using the BLAST program, a region was found in the mouse BAG-1 protein (residues 37 to 73) that has as much as 50% amino acid sequence identity (66% similarity)
25 with ubiquitin and several ubiquitin-like proteins from a variety of species. The ubiquitin-like domain in BAG-1 contains a lysine residue at position 67 that corresponds to lysine-48 of ubiquitin. Lysine-48 in ubiquitin acts as an epsilon-amino acceptor group for covalent ligation to
30 the C-terminal glycine of other ubiquitin molecules during the multi-ubiquitination reactions that occur in proteins targeted for degradation (Rechsteiner, Cell 66:615-618 (1991); Hershko & Ciechanover, Ann. Rev. Biochem. 61:761-807 (1992)).

D. Isolation of a nucleic acid sequence encoding human BAG-1

The ³²P-labelled 833 bp mouse bag-1 cDNA was used as a hybridization probe to screen a human breast cDNA library, which was purchased from Clontech, Inc. Several positive clones were isolated and partially sequenced. The nucleotide sequence of human bag-1 cDNA, including a portion of the coding region of the human BAG-1 protein is shown in Figure 3 (SEQ ID NO: 3). A putative polypeptide derived from the human bag-1 cDNA is shown in Figure 4 (SEQ ID NO: 4).

The human bag-1 cDNA sequences were ³²P-labeled and used to screen a cosmid library prepared from human placental genomic DNA (Stratagene; La Jolla, CA). Two positive cosmid clones, designated TS131-I and TS131-3, were obtained and used as probes to determine the chromosomal location of the human BAG-1 gene using fluorescence *in situ* hybridization as described by Inazawa et al., Genomics 14:821-822 (1992), which is incorporated herein by reference. The human gene mapped to chromosome 9 at approximately band p13 (not shown).

EXAMPLE II

In Vitro Association of BAG-1 and Bcl-2

This example demonstrates that BAG-1 binds Bcl-2 in various *in vitro* assays.

A. Solution binding assay of BAG-1 binding to Bcl-2

The 833 bp cDNA sequence that encodes a portion of the mouse BAG-1 protein was subcloned into the pGEX-3X prokaryotic expression plasmid (Pharmacia; Piscataway, NJ) to produce glutathione-S-transferase (GST)/BAG-1 fusion proteins in *E. coli*. GST-BAG-1 fusion proteins were affinity purified using glutathione-Sepharose (Sigma Chem.

Co.; St. Louis, MO). As a control, GST protein without BAG-1 sequences was produced and immobilized on glutathione-Sepharose. Following loading of the GST-BAG-1 or GST, the columns were washed with solution A to remove
5 irrelevant proteins.

The ability BAG-1 to bind Bcl-2 in solution was examined. GST control or GST-BAG-1 proteins were immobilized on glutathione-Sepharose 4B (0.25 ug protein/ μ l beads) that was preblocked with 0.5% non-fat milk and 0.05%
10 BSA. Bcl-2 protein was produced in Sf9 cells using a bcl-2-containing baculovirus vector as described by Reed et al., *supra*, 1992). Bcl-2-containing lysates were prepared and added in solution A to the glutathione-Sepharose-immobilized GST/BAG-1 or GST proteins.

15 Binding was allowed to proceed at 4°C for 1 hr, then unbound Bcl-2 protein was removed by washing 3x with solution A. The Sepharose particles were collected by centrifugation and boiled in 20 μ l Laemmli sample buffer (62 mM Tris (pH 6.6), 2.3% SDS, 10 % glycerol, 5% 2-
20 mercaptoethanol) to release the bound proteins, then 10 μ l of the sample was size-fractionated by SDS-PAGE (12% gel) and transferred to nitrocellulose filters as described by Reed et al. (Canc. Res. 51:6529-6538 (1991), which is incorporated herein by reference). Immunoblot analysis was
25 performed using 0.2% (v/v) of a rabbit polyclonal antiserum specific for residues 41-54 of the human Bcl-2 (Reed et al., *supra*, 1991) and alkaline-phosphatase-conjugated goat anti-rabbit IgG (Promega). Color detection was accomplished using NBT/BCIP in 0.1 M Tris, pH 9.5, 0.1 M
30 NaCl, 5 mM MgCl₂.

As shown in Figure 6, Bcl-2 protein co-sedimented with the GST-BAG-1-Sepharose particles but not with the GST-Sepharose control. No Bcl-2 protein was detected when GST-BAG-1 was incubated with control Sf9 cell lysates,

which lack Bcl-2, thus confirming the specificity of the results. These results demonstrate that the GST-BAG-1 fusion protein specifically binds Bcl-2.

B. Filter binding assay of BAG-1 binding to Bcl-2

5 BAG-1 also was expressed as a T7/10-fusion protein. pEXlox-BAG-1 was transformed into BL21(DE3) cells, which express T7 RNA polymerase, and grown overnight (Palazzolo et al., supra, 1990; Margolis et al., supra, 1992). The overnight cultures were diluted 1:100 in NZCYM
10 medium containing 100 µg/ml ampicillin and grown at 37 °C for 2 hr with vigorous shaking. IPTG (1 mM final concentration) was added and incubation was continued for 1 to 3 hr. Bacteria were recovered from 1 ml of culture by centrifugation and resuspended in 100 µl boiling Laemmli
15 buffer.

The ability to BAG-1 to bind Bcl-2 was determined using a filter binding assay. Filter binding assays were performed using either 10 ul of BL21(DE) lysate, which contains T7/10-BAG-1 fusion protein, or 0.25 µg purified
20 GST-BAG-1 or GST control proteins. Proteins were size-fractionated by SDS-PAGE and transferred to nitrocellulose filters. The filters were processed as described above for the library screening. Sf9 lysates containing Bcl-2 or β-gal were run in parallel as controls
25 and an anti-T7/10 protein antiserum (Novagen, Inc.; Madison WI) was used to confirm production of T7/10-fusion proteins. The results of the filter binding assays confirmed that GST-BAG-1 fusion proteins, as well as the T7/10-BAG-1 fusion proteins, specifically bind Bcl-2 (not
30 shown).

C. Yeast two-hybrid assay of BAG-1 binding to Bcl-2

The yeast two-hybrid system (Zervous et al., Cell 72:223-232 (1993), which is incorporated herein by reference; Golemis et al., In Curr. Prot. Mol. Biol. (J. Wiley and Sons, Inc. 1994), which is incorporated herein by reference; see pages 13.14.1 to 13.14.17) was employed exactly as described by Sato et al., *supra*, 1994. Plasmid pSN-245-9, containing nucleotides -82 to +843 of the mouse bag-1 cDNA, was employed as a template for PCR amplification using the primers 5'-GAATTCGAGGAGGCGACCCAAAC-3' (forward; SEQ ID NO: 5) and 5'-CCTGGCAGCCATGGAGAAACA-3' (reverse; SEQ ID NO: 6).

Following amplification, the nucleic acids were digested with Eco RI and Nco I and subcloned in-frame with the B42 trans-activation domain in EcoRI/NcoI-digested pJG5-4. The B42-BAG-1 fusion protein was expressed in yeast cells under the control of the galactose-inducible Gal-1 gene promoter. All other constructs in pJG5-4 and in the LexA DNA-binding domain plasmid pEG202 have been described (Sato et al., *supra*, 1994).

Interactions of BAG-1 and Bcl-2 were detected using the lacZ and LEU2 reporter genes containing lexA operators. For these experiments, truncated human Bcl-2 proteins, which lack the transmembrane domain, were expressed in *S. cerevisiae* as fusion proteins with a LexA DNA-binding domain. The truncated Bcl-2 fusion proteins contained amino acids 1 to 218 (LexA-Bcl-2 (1-218)) or 72 to 218 of human Bcl-2.

Activity of lacZ was detected using qualitative β -gal filter assays (Breeden and Nasmyth, Cold Spring Harbor Symp. Quant. Biol. 50:643-650 (1985), which is incorporated herein by reference) or by quantitative β -gal assays using O-nitrophenyl- β -D-galactoside (ONPG) as a

substrate (Reynolds and Lundblad, In Curr. Prot. Mol. Biol. (J. Wiley and Sons, Inc. 1989), which is incorporated herein by reference; see pages 13.6.1 to 13.6.4). Growth on leucine-deficient medium was determined by scoring colony size 5 to 7 days after transformation.

As shown in Figure 7, the B42-BAG-1 fusion protein interacted with the LexA-Bcl-2 (1-218) protein in yeast and trans-activated expression of the lacZ and LEU2 reporter genes (Figure 7). Interaction of B42-BAG-1 with LexA-Bcl-2 produced about a 7 fold increase in β -gal expression above background. In contrast, deletion of N-terminal amino acids 1 to 72 of Bcl-2 (Bcl-2(72-218)) abrogated reactivity with BAG-1. These results indicate that the N-terminal domain of Bcl-2 is required for binding to BAG-1.

Since Bcl-2 is known to form heterodimers, co-expression of LexA-Bcl-2 and B42-Bcl-2 served as a positive control and demonstrated binding activity (Sato et al., *supra*, 1994; Yin et al., Nature 369:321-333 (1994)). Bcl-2 homodimerization produced about a 35 fold increase in β -gal activity above background. In contrast, the B42-BAG-1 protein did not bind to a LexA-Fas protein (Figure 7). β -gal activity and growth on leucine-deficient medium depended on the addition of galactose to the medium; β -gal activity and growth did not occur when cells were plated on glucose-containing medium, which suppresses the Gal-1 promotor (not shown).

EXAMPLE III

BAG-1 Binds to Bcl-X-L, Bcl-X-S and Bcl-2 β

This example demonstrates that BAG-1 binds to various Bcl-2-related proteins *in vitro*.

BAG-1 protein was examined for binding to the two protein forms encoded by bcl-X gene. The two forms of Bcl-X protein, which are produced by alternative splicing, include a 233 amino acid protein, Bcl-X-L, and a 170 amino acid protein, Bcl-X-S (Boise et al., Cell 74:597-608 (1993), which is incorporated herein by reference). Bcl-X-L has 72% homology with Bcl-2 and, like Bcl-2, blocks apoptosis. Bcl-X-S, which lacks a 63 amino acid segment found in Bcl-X-L, also inhibits Bcl-2 function. In addition, the ability of BAG-1 to bind to an alternatively spliced form of Bcl-2, designated Bcl-2 β , which lacks the transmembrane domain and diverges in sequence from Bcl-2 after amino acid 198, was examined (Tsujimoto and Croce, 1986).

The cDNA sequences encoding Bcl-X-L, Bcl-X-S, Bcl-2 and Bcl-2 β were subcloned into plasmid pSK-II (Stratagene), which contains a T7 RNA polymerase binding site. Cloning into pSK-II allows for the *in vitro* production of RNA, which can be translated *in vitro* in the presence of ^{35}S -methionine using reticulocyte lysates to obtain ^{35}S -labeled proteins. The ^{35}S -labeled Bcl-2-related proteins (20 μl of *in vitro* translation mix) were added to approximately 1 μg GST-BAG-1 fusion protein immobilized on 10 μl glutathione-Sepharose or with approximately 1 μg immobilized GST control protein as described above. Following incubation at 4°C for 1 hr, the Sepharose particles were washed 3x in a solution containing 147 mM KCl, 20 mM Hepes (pH 7.1), 1 mM MgCl_2 , 0.5 mM EGTA, 1 mM PMSF, 0.05 NP-40, 1 mM DTT, 1% non-fat dried milk and 1% BSA. The Sepharose particles were collected by centrifugation and boiled in Laemmli sample buffer to release any bound ^{35}S -labeled Bcl-2-related proteins. The released proteins were size-fractionated by SDS-PAGE using a 10% gel and visualized by fluorography using X-ray film.

Bcl-2, Bcl-2 β , Bcl-X-L and Bcl-X-S protein each bound to the GST-BAG-1 fusion protein but not to GST control protein. These results demonstrate that BAG-1 can bind to a variety of Bcl-2-related proteins.

5

EXAMPLE IV

Preparation and Use of Anti-BAG-1 Antibodies

This example describes a method for obtaining polyclonal anti-BAG-1 antibodies and use of the antibodies to identify BAG-1 expression in a tissue.

10 Anti-BAG-1 antibodies were prepared using methods well known in the art (see, for example Harlow and Lane (1988)). Briefly, GST-BAG-1 fusion protein was produced in *E. coli* and affinity purified using glutathione-Sepharose as described above. Approximately 500 μ g of purified
15 protein in Freund's complete adjuvant was injected subcutaneously into New Zealand white rabbits. The original immunization was followed by three weekly booster immunizations using 500 μ g of GST/BAG-1 protein in Freund's incomplete adjuvant. Following the third booster dose,
20 immunizations were administered at 4-6 week intervals; blood samples were collected 1 to 2 weeks after each booster for preparation of sera.

Antibodies that reacted with the GST protein were removed by passing a 1:10 (v:v) solution of rabbit
25 antiserum in phosphate buffer saline (PBS) through a column containing GST protein immobilized on glutathione Sepharose as described in Examples II.A., above. The effluent was passed through a second column containing GST/BAG-1-Sepharose and specific antibodies were eluted using 0.2 M
30 glycine (pH 2.2). The sample pH was adjusted to pH 7.4 and dialyzed against PBS containing 0.2% NaN₃.

Antisera also were raised to a synthetic peptide representing the C-terminal 16 amino acids of the mouse BAG-1 protein (see Figure 2). The peptide H₂N-CQETERLQSTNLALAE-COOH (SEQ ID NO: 7) was synthesized and
5 contains at its amino terminus a cysteine residue, which was used to covalently conjugate the peptide to a carrier protein, maleimide-activated KLH, as described by Reed et al. (1991). The KLH-peptide conjugates were used to immunize rabbits as described above.

10 The anti-BAG-1 antibodies were used for immunoblotting, immunoprecipitation and immunocytochemistry experiments as described below (see, also, Hanada et al., Canc. Res. 53:4978-4986 (1993); Reed et al. (1991); Louie et al., Amer. J. Pathol. 139:1231-1237 (1991), each of
15 which is incorporated herein by reference. Standard immunoblot and immunoprecipitation assays were performed as described by Reed et al., *supra*, (1991), except that blots were preblocked in TBS (10 mM Tris, pH 7.5, 150 mM NaCl) containing 3% BSA and 5% non-fat milk and washes were
20 performed using TBS containing 0.05% Triton-X100™. Antibodies to human and mouse Bcl-2 were prepared as described by Miyashita et al., Oncogene 9:1799-1895 (1994); Krajewski et al., Amer J. Pathol. 145:515 (1994), each of which is incorporated herein by reference, and Reed et al.,
25 *supra*, 1991. For immunocytochemistry, mouse tissues were fixed in Bouin's solution, embedded in paraffin, sectioned and immunostained using a diaminobenzidine colorimetric detection method as described by Miyashita et al., *supra*, 1994, and Krajewski et al., *supra*, 1994.

30 The anti-BAG-1 antisera detected a protein having an apparent molecular weight of approximately 30 kDa (not shown), which is somewhat larger than the molecular weight of 24,846 Da predicted from the 219 amino acid open reading frame present in the mouse BAG-1 cDNA (Figure 1). This
35 discrepancy likely is due to the highly acidic nature of

the BAG-1 protein (14% glutamic acid), which can result in aberrant migration in SDS-containing polyacrylamide gels. The aberrant mobility of the BAG-1 protein was confirmed by demonstrating that electrophoresis of a protein prepared by
5 *in vitro* transcription and translation of a cDNA that lacks the 5'-untranslated region and contains only the open reading frame coding for the 219 amino acid polypeptide had the same migration.

Immunoblot and immunocytochemical detection of
10 BAG-1 protein demonstrated that the protein is present in a wide variety of tissues (not shown). BAG-1 is most abundant in thymus, spleen and lymph nodes, moderately abundant in brain, and minimally detectable in kidney, skeletal muscle and liver.

15 Intracellular localization of BAG-1 and Bcl-2 also was determined by light microscopy. Similar patterns of expression were observed for BAG-1 and Bcl-2 (not shown). For example, sequential 5 μ m sections through mouse intestinal villi demonstrated that BAG-1 was
20 localized primarily in the cytosol. The pattern of immunostaining was not homogeneous, indicating that BAG-1 is present in association with supranuclear structures that probably represent organelles. The pattern of Bcl-2 immunostaining in these intestinal epithelial cells was
25 very similar (not shown). Preimmune serum exhibited negligible immunoreactivity under the conditions used.

EXAMPLE V

BAG-1 and Bcl-2 Associate *In Vivo*

This example demonstrates that BAG-1 and Bcl-2
30 form a bound complex *in vivo*.

Anti-BAG-1 antibodies were used to show that Bcl-2 protein co-immunoprecipitates with BAG-1. A murine

cell line, 32D, was transfected with either a bcl-2 expression plasmid, pZip-bcl-2, which produces the human Bcl-2 protein, or with a negative control plasmid, pZip-neo. The cell lines were designated 32D-BCL-2 and
5 32D-NEO, respectively (see Baffy et al., J. Biol. Chem. 268:6511-6519 (1993); Tanaka et al., J. Biol. Chem. 268:10920-10926 (1993), each of which is incorporated herein by reference).

32D cells were lysed in a lysis buffer containing
10 147 mM KCl, 20 mM Tris (pH 7.5), 2.5 mM MgCl₂, 0.05 mM EGTA, 0.5% NP-40 and protease inhibitors. Approximately 500 µg of the resulting proteins were incubated with 5 µl of anti-BAG-1 antiserum for 2 hr at 4°C. Following incubation, the immune complexes were added to 25 µl of
15 protein A-Sepharose. The sample was washed 3x with the lysis buffer and the Sepharose particles were pelleted by centrifugation and boiled in Laemmli sample buffer. The proteins were size-fractionated by SDS-PAGE and transferred to nitrocellulose filters. The filters were incubated with
20 5-10 ml phosphate buffered saline (pH 7.4) containing 2% (w/v) BSA and 0.1% (v/v) of a rabbit antiserum specific for the human Bcl-2 protein, then with horseradish peroxidase-conjugated goat-anti-rabbit IgG and the LumiphosTM substrate (Boehringer Mannheim, Inc.) as
25 suggested by the supplier. Chemiluminescence was detected by autoradiography.

A 26 kDa protein corresponding to the human Bcl-2 protein was detected in lysates derived from 32D-BCL-2 cells, which produce the human Bcl-2 protein, but not in
30 lysates prepared from 32D-NEO cells, which lack human Bcl-2 protein. These results indicate that Bcl-2 and BAG-1 associate in cells *in vivo*.

EXAMPLE VI

The Raf-1 Kinase Associates with Bcl-2 in vivo

This example demonstrates that a Raf-related protein, the Raf-1 kinase, has the characteristics of a Bcl-2-associated protein.

To facilitate detection of the Raf-1 kinase interaction with Bcl-2, an expression plasmid, pEc12 (Heidecker et al., *supra*, (1990), which produces an amino-terminal truncated, constitutively active 35 kDa form of the Raf-1 kinase, was introduced in either 32D-BCL-2 cells or 32D-NEO cells, as described above. Production of the 35 kDa Raf-1 kinase was confirmed by immunoblot analysis using a polyclonal antiserum raised against a synthetic peptide corresponding to the carboxy-terminal 16 amino acids of Raf-1 as described by Reed et al., Cell Growth Diff. 2:235-243 (1991b), which is incorporated herein by reference.

Ten million of each of the resulting 32D-BCL-2/Raf cells and 32D-NEO/Raf cells were lysed in a solution containing 147 mM KCl, 20 mM Tris (pH 7.5), 2.5 mM MgCl₂, 0.05 mM EGTA, 0.5% NP-40 and protease inhibitors. Bcl-2 protein was immunoprecipitated using 2.5 μ l of anti-Bcl-2 polyclonal antiserum and 25 μ l of protein A-Sepharose. The samples were washed 3x with lysis solution and boiled in Laemmli sample buffer. Proteins were size-fractionated by SDS-PAGE (10% gel) and transferred to nitrocellulose filters. The resulting blots were incubated with 0.1% (v/v) of the Raf-1-specific antiserum in 5 to 10 ml of phosphate buffered saline containing 2% BSA. Bound antibodies were detected using biotinylated goat anti-rabbit IgG (Vector Labs, Inc.) and an avidin-biotin complex reagent containing horseradish peroxidase (Vector Labs, Inc.), followed by diaminobenzidine for color detection essentially as described by Hanada et al. (1993).

A 35 kDa band corresponding to the truncated Raf-1 kinase was detected by this immunoblot analysis of Bcl-2-containing immune complexes prepared from 32D-BCL-2/Raf cells. No band was observed in the lane corresponding to
5 lysate prepared from 32D-NEO/Raf cells, reflecting that these cells do not express human Bcl-2 and that the anti-Bcl-2 antibody is specific for human Bcl-2 (Reed et al. (1991). These results indicate that the truncated 35 kDa form of Raf-1 can associate with Bcl-2 *in vivo*.

10 The ability of the full length Raf-1 kinase to bind Bcl-2 also was examined. Instead of immunoblot analysis, a more sensitive *in vitro* immune complex kinase assay was used (Reed et al., 1991b). Lysates were prepared from 32D-BCL-2 cells, which overexpress human Bcl-2 protein
15 and express endogenous Raf-1 kinase. Immunoprecipitation assays were performed using either an anti-Bcl-2 monoclonal antibody, 4D7 (Reed et al. (1992), or an isotype and subclass-matched control mouse IgG₁ monoclonal antibody (Sigma Chem. Co.). Immune complexes were washed and
20 resuspended in a kinase reaction buffer containing γ -³²P-ATP and a peptide substrate exactly as described by Reed et al. (1991b). Following incubation for 20 min at 30°C, the peptide substrate was separated from unincorporated γ -³²P-ATP by SDS-PAGE using a 20% gel and the relative amount of
25 incorporated ³²P was determined by autoradiography.

Immune complexes prepared using the Bcl-2-specific monoclonal antibody contained 20x to 50x more Raf-1 kinase activity than control immune complexes. This result indicates that the full length endogenous Raf-1
30 kinase can associate with protein complexes containing Bcl-2 protein.

EXAMPLE VII

BAG-1 Expression Reduces Apoptosis in a Cell

This example demonstrates that BAG-1 expression in Jurkat T cell acute lymphocytic leukemia cells and mouse
5 Balb/c-3T3 fibroblasts reduces the sensitivity of these cell to apoptosis.

A. Jurkat T cells

Gene transfer experiments were used to examine the function of BAG-1 alone or in combination with Bcl-2.
10 The bag-1 cDNA insert was excised from pSN-245-9 using Hind III and Bam HI and subcloned into pCEP-4 (Invitrogen, Inc.), which contains the constitutive CMV immediate-early region promotor and a gene encoding hygromycin-phosphotransferase (pCEP-BAG-1). Jurkat cells first were
15 stably transfected with pZip-Bcl-2 (Jurkat-Bcl-2 cells), which expresses Bcl-2, or a control pZip-Neo (Jurkat-Neo cells) expression plasmid and selected in G418 (Torigoe et al., Canc. Res. 54:4851-4854 (1994a); Torigoe et al., J. Exp. Med. 180:1115-1127 (1994b), which is incorporated
20 herein by reference).

Jurkat-Bcl-2 and Jurkat-Neo cells (5×10^6) were mixed on ice with 25 μ g pCEP-BAG-1 or the control pCEP-4 plasmid in 0.8 ml HBS (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 6 mM dextrose) and transfected by
25 electroporation (270 volts, 900 μ F) in 0.4 cm (diameter) cuvettes with platinum electrodes (CellJect; EquiBio, Inc.; BIOS, Inc.; New Haven CT). The transfected cells were incubated in RPMI for 2 days, then 1×10^5 cells were seeded/ml RPMI containing 10% FCS, 1 mM L-glutamine, 100
30 U/ml penicillin G, 50 μ g/ml streptomycin and 1 mg/ml hygromycin (Calbiochem) and passaged as described by Reed et al. (Mol. Cell. Biol. 10: 4370 (1990c), which is

incorporated herein by reference). Individual clones were obtained by limiting dilution.

Immunoblot analysis of the double transfected Jurkat cells using anti-BAG-1 and anti-Bcl-2 antisera revealed that elevated levels of BAG-1 protein were present in the Jurkat-Bcl-2 and Jurkat-Neo transfectants that also were transfected with pCEP-BAG-1 but not those cells that were transfected with the control pCEP-4 plasmid (not shown). Bcl-2 protein levels also were markedly elevated in the Jurkat-Bcl-2 cells as compared to the Jurkat-Neo cells (not shown).

The relative sensitivity of the double transfected Jurkat cells to induction of apoptosis by an anti-Fas monoclonal antibody, 2D1 (Takahashi et al., Eur. J. Immunol. 23:1935-1941 (1993), which is incorporated herein by reference), which can trigger apoptosis via Fas antigen; by 10 μ M staurosporine, a general protein kinase inhibitor; by a panel of CTL clones that induce target cell killing through mechanisms consistent with apoptosis; or by buthionine sulfoximine (BSO), which depletes intracellular glutathione (Torigoe et al., *supra*, 1994a, 1994b), was examined. Since elevated levels of Lck kinase activity enhance CTL killing, some of the experiments performed with CTL's that had been stably transfected to express the normal Lck kinase or a constitutively active mutant Lck (Torigoe et al., *supra*, 1994a, 1994b).

To induce cytotoxicity, Jurkat cells were cultured in 96-well flat-bottom plates (0.2 ml/well; Falcon) for various times at 5×10^5 cells/ml RPMI medium containing 10% FCS, 1 mM L-glutamine, 100 U/ml penicillin G, 50 μ g/ml streptomycin and either 1 μ g/ml 2D1 antibody, 10 μ M staurosporine (Sigma) or 10 mM L-buthionine sulfoximine (Sigma). Relative numbers or percentages of viable cells were determined by trypan blue dye exclusion

or by the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay (Kitada et al., Antisense Res. Devel. 4:71-79 (1994); Tada et al. J. Immunol. Meth. 93:157-165 (1986), each of which is
5 incorporated herein by reference). The MTT assays were confirmed to be in the linear range and standard curves were run in parallel in each assay.

As shown in Figure 8.A., Jurkat cells having gene transfer-mediated elevations in Bcl-2 protein were
10 partially resistant to cytotoxicity induced by 1 ug/ml anti-Fas antibody. This result is consistent with a previous report using other T cell lines transfected with bcl-2 expression plasmids (Itoh et al., J.Immunol. 151:612-627 (1993)). The morphological changes induced by the
15 anti-Fas antibody showed the characteristic cell morphology and oligonucleosomal pattern of genomic DNA fragments associated with apoptosis (not shown).

In contrast to Bcl-2, gene transfer-mediated elevations in BAG-1, alone, had little if any effect on
20 anti-Fas antibody-induced cytotoxicity of Jurkat cells. However, the combination of elevated BAG-1 and Bcl-2 protein levels produced markedly enhanced resistance to anti-Fas antibody-induced apoptosis (Figure 8.A.). In the experiment shown in Figure 8.A., for example, greater than
25 80% of the Jurkat cells transfected with both Bcl-2 and BAG-1 remained viable up to 2 days after treatment with 1 ug/ml anti-Fas antibody. In comparison, about 25% of the Jurkat cells having elevated levels of Bcl-2, alone, remained viable and less than 1% of the Jurkat cells
30 transfected with pCEP-BAG-1, alone, were viable. Similar results were observed in other experiments (not shown).

Similar results also were obtained when apoptosis was induced by treatment of the Jurkat cells with the protein kinase inhibitor staurosporine. As shown in Figure

8.B., expression of BAG-1, alone, provided only slightly enhanced protection from staurosporine-induced cell death. Bcl-2 was more protective, although fewer than half of the cells remained alive after 2 days. Again, however, the
5 combination of BAG-1 and Bcl-2 afforded marked resistance to staurosporine-induced cell death, with about 80% of the cells surviving exposure to 10 μ M staurosporine for 2 days.

Finally, the effect of gene transfer-mediated elevations in BAG-1 protein levels on susceptibility to
10 killing by cytolytic T lymphocytes (CTL's) was examined. The cloned CTL line used in these studies exhibits lymphokine-activated killer cell-like cytolytic activity against Jurkat cells and certain other tumor cell lines through a CD3- and MHC-independent mechanism (Torigoe et
15 al., *supra*, 1994a, 1994b). The CTL cytolytic activity was enhanced by gene transfer-mediated elevations in Lck kinase activity.

For these experiments, Jurkat cells were labeled with chromium-51 and cultured for 4 hr with CTL's at an
20 effector cell:target cell ratio of 20:1. The percentage specific chromium-51 release was calculated by correcting for spontaneous release of chromium-51 and normalizing for total release induced by 1% NP-40 as described by Torigoe et al., *supra*, 1994a, 1994b.

25 The variously transfected Jurkat cells were employed as targets for CTL cytolytic assays. As shown in Figure 8.C., Jurkat cells that expressed elevated BAG-1 protein exhibited a slight but reproducible ($n = 3$) resistance to lysis induced by control CTL's (Neo) as well
30 as by CTL's that over-express the normal Lck kinase (N-LCK) or activated Lck kinase (A-LCK). Jurkat target cells expressing elevated levels of Bcl-2 protein were relatively more resistant than the BAG-1 transfectants but were not as

resistant as Jurkat cells expressing both the Bcl-2 and BAG-1 proteins (Figure 8.C.).

As compared to control transfected cells, Jurkat cells that over-expressed both Bcl-2 and BAG-1 showed about
5 16% to 23% the amount of lysis. Jurkat cells expressing only BAG-1 showed about 76% to 79% lysis as controls and Jurkat cells expressing only Bcl-2 showed about 40% to 50% lysis as compared to controls. These results indicate that, in Jurkat T cells, BAG-1, alone, has relatively
10 little effect on reducing cell death. In combination with Bcl-2, however, BAG-1 markedly enhanced resistance to apoptosis induced by several stimuli.

The above described experiments used mixed populations of transfected Jurkat cells. To more carefully
15 examine the effect of Bcl-2 and BAG-1, cloned cell lines were obtained from the mixed populations and similar experiments were performed. Several clones of Jurkat cells expressing both Bcl-2 and BAG-1 were isolated. All of the cloned cell lines expressed nearly equivalent levels of
20 BAG-1 protein and of Bcl-2 protein as determined by immunoblot analysis. Similar results were obtained from clones of Jurkat cells transfected with pCEP-BAG-1, alone, or pZIP-Bcl-2, alone.

Figure 9 shows the results for two representative
25 clones, designated 5-2 and 7-2. Clone 5-2 was obtained from Jurkat cells transfected to express both BAG-1 and Bcl-2, whereas clone 7-2 was from cells transfected to express only Bcl-2. Both clones expressed comparable levels of Bcl-2 but only clone 5-2 over-expressed BAG-1
30 (compares Figure 9.A. and 9.B.). Despite expressing nearly equivalent levels of Bcl-2 as clone 7-2 cells, clone 5-2 cells displayed significantly enhanced survival following exposure to anti-Fas antibody (Figure 9.C.) and staurosporine (not shown). 5-2 cells also were markedly

more resistant to induction of cell death by BSO (Figure 9.D.). These results confirm that BAG-1 can augment the anti-cell death function of Bcl-2 in Jurkat T cells.

B. Balb/c-3T3 fibroblasts

5 The effect of BAG-1 expression on cell survival also was examined in Balb/c-3T3 mouse fibroblast cells.

 The bag-1 cDNA insert was excised from pSN-245-9 using Hind III and Xba I and subcloned into pRc/CMV (Invitrogen), which contains the CMV promotor described
10 above and a gene encoding neomycin phosphotransferase (pRc/CMV-BAG-1). The pRc/CMV plasmid integrates into genomic DNA following transfection.

 pRc/CMV-BAG-1 or pRc/CMV control plasmid was linearized using Sca I and 25 μ g DNA was mixed on ice with
15 2.5 x 10⁶ Balb/c-3T3 cells in 0.8 ml HBS. Linearized plasmid DNA was transfected into 3T3 cells by electroporation (270 volts, 1500 μ F) as described above. Cells were allowed to incubate for 2 days, then selected in DMEM containing 1 mg/ml geneticin (G418; Gibco/BRL).
20 Individual clones were isolated using cloning cylinders as described by Reed et al., *supra*, 1990c). For comparison, 3T3 cells also were transfected with a pZIP-Bcl-2 or control pZIP-Neo expression plasmids.

 Initially, a polyclonal line of G418-resistant
25 BAG-1-transfected 3T3 cells was obtained. However, the level of BAG-1 protein in these cells was not detectably different than the level of BAG-1 in 3T3 cells transfected with the control pRc/CMV plasmid (not shown). Individual clones were obtained and, when examined for BAG-1 protein
30 levels by immunoblot analysis, showed variable levels of BAG-1 expression (not shown). The differences in

expression may reflect different sites of integration of the plasmid DNA in the genome.

One clone (clone 19), which contained about 3 fold higher levels of BAG-1 than the control 3T3 transfectants, was used to determine the effect of elevated BAG-1 expression on cell survival. For comparison, a second clone (clone 28), which had been transfected with pRc/CMV-BAG-1 but did not express elevated levels of BAG-1, was examined in parallel. The effect of staurosporine, which induces apoptosis in fibroblast cell lines (Jacobson et al., Nature 361:365-368 (1993)), was examined.

Clone 19 and clone 28 cells, as well as 3T3 cells transfected with pZIP-Bcl-2 or pZIP-Neo, were incubated in the presence of 1 μ M staurosporine and cell survival was determined at various times. As shown in Figure 10.A., the gene transfer-mediated elevation of BAG-1 expression in clone 19 cells prolonged the ability of these cells to survive following exposure to staurosporine as compared to the survival of clone 28 cells, which did not produce an elevated level of BAG-1 protein. Similar results were obtained when other clones having elevated or normal levels of BAG-1 were examined (not shown). Furthermore, the increased cell survival observed for clones having gene transfer-mediated elevations in BAG-1 protein was similar to the increased survival observed in 3T3 cells that were stably transfected with pZIP-Bcl-2 and shown by immunoblot assay to contain substantial amounts of Bcl-2 protein (Figure 10.B.). These results demonstrate that, in some cell types, increased expression of BAG-1, alone, can enhance cell survival without the need for increasing the amount of Bcl-2 expression.

EXAMPLE VIII

Drug Screening Assays

This example describes assays that are useful for screening for agents such as drugs that alter the affinity of binding of BAG-1 with Bcl-2.

Figure 11 presents a scheme for using a BAP such as the BAG-1 protein or the Raf-1 kinase in a drug screening assay that is suitable for automated high through-put random drug screening. A cDNA encoding BAG-1 was subcloned into either the pGEX-3X plasmid, which produces GST-BAG-1 fusion proteins in *E. coli*, as described above, or into a baculovirus transfer vector, pAcSG-His, which produces histidine-tagged fusion proteins in Sf9 insect cells (PharMingen, Inc.). The proteins were affinity purified by standard methods using either glutathione-Sepharose, as described above, or nickel-chelation chromatography, essentially as described by Smith and Johnson, Gene 67:31-40 (1988), which is incorporated herein by reference. The specific recombinant fusion proteins were eluted using excess glutathione in PBS (pH 7.4) or in imidazole (pH 6.0), respectively. Following dialysis, the GST-BAG-1 and His-BAG-1 fusion proteins were immobilized to solid supports taking advantage of the ability of the GST protein to specifically bind glutathione and of the histidine-6 peptide region to chelate nickel (Ni) ion.

The assay can utilize any Bcl-2-related protein, including a truncated form of the Bcl-2 protein that lacks the hydrophobic transmembrane domain, which obviates problems of solubility. The truncated protein was engineered to contain a mutation of Cys->Ala at amino acid position 158 of the human Bcl-2 protein. As a result of this mutation, the protein contained only one cysteine residue and, therefore, has a free sulfhydryl (SH) group

available for chemical modification. The 158 Cys->Ala mutation does not impair the biological activity of the Bcl-2 protein when expressed in mammalian cells. These results indicate that the mutant Bcl-2 protein folds
5 correctly and retains its ability to interact with relevant proteins in cells.

Since the mutant Bcl-2 protein has a single free sulfhydryl group located at its carboxy terminus, several different chemical modifications can be used to attach a
10 detectable moiety such as a fluorescent molecule, a radiolabel or another protein, which can be detected using a specific antibody or other specific reagent. For example, fluorescein-5 maleimide can be attached as a fluorescent tag for the Bcl-2 protein. Various agents such
15 as drugs are screened for the ability to alter the association of Bcl-2 and BAG-1. The agent, BAG-1 and fluorescent-Bcl-2 can be added together, incubated for 30 min to allow binding, then washed to remove unbound fluorescent-Bcl-2 protein. The relative amount of binding
20 of fluorescent-Bcl-2 protein in the absence as compared to the presence of the agent being screened is determined by detecting the relative light emission of the fluorochrome.

The assay is readily adapted for examining the interaction of a BAP with other Bcl-2-related proteins such
25 as Bcl-X-L, Bcl-X-S, Mcl-1, BHRF-1 or Al, as described above, and other Bcl-2-related proteins such as Bax (Oltvai et al., *supra*, 1993) or LMH-5W (Neilan et al., J. Virol. 67:4391-4394 (1993), which is incorporated herein by reference). The screening assay is useful for detecting
30 agents that alter the association of a BAP and a Bcl-2-related protein by increasing or decreasing their binding affinity.

In addition, the drug screening assay is readily adaptable for identifying the effect of an agent on the

binding of other BAP proteins such as the Raf-1 kinase with a Bcl-2-related protein. In this case, it can be advantageous to include in the assay a third protein such as a member of the Ras family of proteins, which are known to interact with the Raf kinase (Vojtek et al., Cell 74:205-214 (1993), which is incorporated herein by reference). The addition of the Ras protein can facilitate the interaction of Raf-1 with a Bcl-2-related protein *in vitro* and will result in an assay that more closely mimics the interactions that occur in the intracellular environment.

The two-hybrid assay also is useful as a drug screening assay and can be performed as described in Example II.C. For example, a BAG-1-LexA hybrid and a Bcl-2-B42 hybrid can be expressed in a cell such as a yeast cell, provided that the cell contains a reporter gene and that the hybrids can bind to each other to activate transcription of the reporter gene.

The cells can be incubated in the presence of an agent suspected of being able to alter the binding of the hybrids to each other. An agent such as a drug that effectively alters an interaction of the hybrids can be identified by an increase or decrease, for example, in the intensity of the blue color produced due to transcription of a lacZ reporter gene. A control level of binding can be determined by identifying the level of transcription in the absence of the agent. Quantitative β -gal assays also can be performed as described by Reynolds and Lundblad, *supra*, 1989.

The screening assay is particularly useful for screening a panel of agents to identify an effective agent. For screening a panel of agents, the assay can be performed in parallel in 96 well plates. Following incubation in the absence or presence of various agents or combinations of

agents for an appropriate time, cell extracts can be prepared and β -gal activity can be determined using either a filter assay or a soluble β -galactosidase assay using cell lysates as described in Example II.C. Agents that
5 effectively increase or decrease, as desired, binding of the hybrids can be identified by simple visual inspection of the filter or by quantitative spectrophotometry and effective agents can be selected.

Although the invention has been described with
10 reference to the examples above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Reed, John C.
Takayama, Shinichi
Sato, Takaaki
- (ii) TITLE OF INVENTION: Bcl-2-associated proteins
- (iii) NUMBER OF SEQUENCES: 7
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: CAMPBELL AND FLORES
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 - (C) CITY: San Diego
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 - (E) COUNTRY: USA
 - (F) ZIP: 92122
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/152,485
 - (B) FILING DATE: 12-NOV-1993
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Campbell, Cathryn A.
 - (B) REGISTRATION NUMBER: 31,815
 - (C) REFERENCE/DOCKET NUMBER: FP-LJ 1201
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (619) 535-9001
 - (B) TELEFAX: (619) 535-8949

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1054 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

| | |
|---|-----|
| CGCCTGCAAG CCGCGGGTGA AGAAGAAAGT CCGCCCCCGC TCTTCTCAGA GCGAGAAGGT | 60 |
| AGGGAGCAGC AGCAGGGAGT TGACTAGAAG TAAGAAAGTG ACCCGTAGCA AGAACGTGAC | 120 |
| CGGGACCCAG GTAGAGGAGG TGACCAAGAT CGAGGAGGCG ACCCAAACCG AGGAAGTAAC | 180 |
| TGTGGCAGAA GAGGTGACCC AGACCGACAA CATGGCCAAG ACCGAGGAGA TGGTCCAGAC | 240 |

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GGAGGAAATG GAAACACCCA GACTCAGCGT GATCGTCACC CACAGCAATG AGAGGTATGA      300
CCTTCTTGTT ACCCCACAGC AAGGTAACAG TGAGCCAGTT GTCCAAGACT TGGCTCAGCT      360
TGTTGAAGAG GCCACAGGAG TTCCACTACC TTTTCAGAAG CTCATATTTA AGGGAAAATC      420
TCTGAAAGAA ATGGAAACAC CGTTGTCAGC ACTTGGAATG CAAAATGGTT GCCGAGTCAT      480
GTTAATTGGT GAAAAGAGCA ATCCAGAAGA AGAGGTTGAG TTAAAGAAGC TGAAAGATTT      540
GGAGGTATCT GCAGAGAAGA TAGCTAACCA CCTGCAAGAA TTGAATAAAG AGCTTTCTGG      600
CATCCAGCAG GGTTTTCTGG CTAAGGAATT GCAAGCGGAG GCTCTCTGCA AACTTGATAG      660
GAAAGTAAAA GCAACAATTG AGCAATTCAT GAAGATCTTG GAGGAGATTG ACACAATGGT      720
CCTACCAGAA CAATTTAAAG ACAGCAGGCT AAAAAGGAAG AATTTGGTGA AAAAGGTTCA      780
GGTGTCTTTA GCAGAGTGTG ACACAGTGGA GCAATACATC TGCCAAGAGA CAGAGCGGCT      840
GCAGTCTACA AACTTGGCCC TGGCTGAATG AAGTGCAGTG GAGAGTGGCT GTACTGGCCT      900
GAAGAGCAGC TTTACAGCCC TGCCCTCTCT GGAACAGAAG TCGCCTGTTT CTCCATGGCT      960
GCCAGGGGCA ACTAGCCAAA TGTCAATTTC CCTGCTCCTC CGTCGGTTCT CAATGAAAAA     1020
GTCCTGTCTT TGCAACCTGA AAAAAAAAAA AAAA                                     1054

```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 219 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Ala Lys Thr Glu Glu Met Val Gln Thr Glu Glu Met Glu Thr Pro
1          5          10          15
Arg Leu Ser Val Ile Val Thr His Ser Asn Glu Arg Tyr Asp Leu Leu
20          25          30
Val Thr Pro Gln Gln Gly Asn Ser Glu Pro Val Val Gln Asp Leu Ala
35          40          45
Gln Leu Val Glu Glu Ala Thr Gly Val Pro Leu Pro Phe Gln Lys Leu
50          55          60
Ile Phe Lys Gly Lys Ser Leu Lys Glu Met Glu Thr Pro Leu Ser Ala
65          70          75          80
Leu Gly Met Gln Asn Gly Cys Arg Val Met Leu Ile Gly Glu Lys Ser
85          90          95
Asn Pro Glu Glu Val Glu Leu Lys Lys Leu Lys Asp Leu Glu Val
100         105         110
Ser Ala Glu Lys Ile Ala Asn His Leu Gln Glu Leu Asn Lys Glu Leu
115         120         125

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Ser Gly Ile Gln Gln Gly Phe Leu Ala Lys Glu Leu Gln Ala Glu Ala
 130 135 140

Leu Cys Lys Leu Asp Arg Lys Val Lys Ala Thr Ile Glu Gln Phe Met
 145 150 155 160

Lys Ile Leu Glu Glu Ile Asp Thr Met Val Leu Pro Glu Gln Phe Lys
 165 170 175

Asp Ser Arg Leu Lys Arg Lys Asn Leu Val Lys Lys Val Gln Val Phe
 180 185 190

Leu Ala Glu Cys Asp Thr Val Glu Gln Tyr Ile Cys Gln Glu Thr Glu
 195 200 205

Arg Leu Gln Ser Thr Asn Leu Ala Leu Ala Glu
 210 215

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 733 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

| | |
|--|-----|
| CCTTCATGTT ACCTCCCAGC AGGGCAGCAG TGAACCAGTT GTCCAAGACC TGGCCCAGGT | 60 |
| TGTTGAAGAG GTCATAGGGG TTCCACAGTC TTTTCAGAAA CTCATATTTA AGGGAAAATC | 120 |
| TCTGAAGGAA ATGGAAACAC CGTTGTCAGC ACTTGGAATA CAAGATGGTT GCCGGGTCAT | 180 |
| GTTAATTGGG AAAAAGAACA GTCCACAGGA AGAGGTTGAA CTAAAGAAGT TGAACATTT | 240 |
| GGAGAAGTCT GTGGAGAAGA TAGCTAACCA GCTGGAAGAG TTGAATAAAG AGCTTACTGG | 300 |
| AATCCAGCAG GGTTCCTGTC CCAAGGATTT GCAAGCTGAA GCTCTCTGCA AACTTGATAG | 360 |
| GAGAGTAAAA GCCACAATAG AGCAGTTTAT GAAGATCTTG GAGGAGATTC ACACACTGAT | 420 |
| CCTGCCAGAA AATTTCAAAG ACAGTAGATT GAAAAGGAAA GGCTTGGTAA AAAAGGTTCA | 480 |
| GGCATTCCCTA GCCGAGTGTG ACACAGTGGA GCAGAACATC TGCCAGGAGA CTGAGCGGCT | 540 |
| GCAGTCTACA AACTTTGCCC TGGCCGAGTG AGGTGTAGCA GAAAAAGGCT GTGCTGCCCT | 600 |
| GAAGAATGGC GCCACCAGCT CTGCCGTCTC TGGATCGGAA TTTACCTGAT TTCTTCAGGG | 660 |
| CTGCTGGGGG CAACTGGCCA TTTGCCAATT TTCCTACTCT CACACTGGTT CTCAATGAAA | 720 |
| AATAGTGTCT TTG | 733 |

60

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 189 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Leu His Val Thr Ser Gln Gln Gly Ser Ser Glu Pro Val Val Gln Asp
 1           5           10           15
Leu Ala Gln Val Val Glu Glu Val Ile Gly Val Pro Gln Ser Phe Gln
 20           25           30
Lys Leu Ile Phe Lys Gly Lys Ser Leu Lys Glu Met Glu Thr Pro Leu
 35           40           45
Ser Ala Leu Gly Ile Gln Asp Gly Cys Arg Val Met Leu Ile Gly Lys
 50           55           60
Lys Asn Ser Pro Gln Glu Glu Val Glu Leu Lys Lys Leu Lys His Leu
 65           70           75           80
Glu Lys Ser Val Glu Lys Ile Ala Asn Gln Leu Glu Glu Leu Asn Lys
 85           90           95
Glu Leu Thr Gly Ile Gln Gln Gly Phe Leu Pro Lys Asp Leu Gln Ala
100          105          110
Glu Ala Leu Cys Lys Leu Asp Arg Arg Val Lys Ala Thr Ile Glu Gln
115          120          125
Phe Met Lys Ile Leu Glu Glu Ile His Thr Leu Ile Leu Pro Glu Asn
130          135          140
Phe Lys Asp Ser Arg Leu Lys Arg Lys Gly Leu Val Lys Lys Val Gln
145          150          155          160
Ala Phe Leu Ala Glu Cys Asp Thr Val Glu Gln Asn Ile Cys Gln Glu
165          170          175
Thr Glu Arg Leu Gln Ser Thr Asn Phe Ala Leu Ala Glu
180          185

```

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 23 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAATTCGAGG AGGCGACCCA AAC

23

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCTGGCAGCC ATGGAGAAC A

21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 16 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Cys Gln Glu Thr Glu Arg Leu Gln Ser Thr Asn Leu Ala Leu Ala Glu
1 5 10 15

We claim:

1. A nucleic acid molecule, comprising the Bcl-2-associated athanogene-1 (bag-1), which encodes a polypeptide or an active fragment thereof, which binds to
5 a Bcl-2-related protein.

2. The nucleic acid molecule of claim 1, wherein said Bcl-2-related protein is Bcl-2.

3. The nucleic acid molecule of claim 1, wherein said Bcl-2-related protein is selected from the group
10 consisting of Bcl-X-L, Bcl-X-S and Bcl-2 β .

4. The nucleic acid molecule of claim 1, wherein said molecule encodes a mammalian BAG-1.

5. The nucleic acid molecule of claim 4, wherein said mammal is a human.

15 6. The nucleic acid molecule of claim 5, comprising the nucleotide sequence of Figure 3 (SEQ ID NO: 3) or an equivalent sequence encoding BAG-1 or an active fragment thereof.

7. The nucleic acid molecule of claim 4, wherein
20 said mammal is a mouse.

8. The nucleic acid molecule of claim 7, comprising the nucleotide sequence comprises of Figure 1 (SEQ ID NO: 1) or an equivalent sequence encoding BAG-1 or an active fragment thereof.

25 9. A probe, comprising a nucleotide sequence that hybridizes under relatively stringent hybridization conditions to the nucleic acid molecule of claim 1.

10. A nucleic acid molecule, comprising a nucleotide sequence encoding a Bcl-2-associated protein (BAP) or an active fragment thereof, which binds to a Bcl-2-related protein, wherein said BAP is not an antibody, a
5 Bcl-2-related protein, a Raf-related protein or a Raf kinase.

11. A polypeptide, comprising a BAP or an active fragment thereof, which binds to a Bcl-2-related protein, wherein said BAP is not an antibody, a Bcl-2-related
10 protein, a Raf-related protein or a Raf kinase.

12. The polypeptide of claim 11, wherein said BAP or said active fragment thereof binds to Bcl-2.

13. The polypeptide of claim 11, wherein said BAP or said active fragment thereof binds to a Bcl-2-
15 related protein selected from the group consisting of Bcl-X-L, Bcl-X-S and Bcl-2 β .

14. The BAP of claim 11, comprising BAG-1 or an active fragment thereof, which can bind to a Bcl-2-related protein.

20 15. The BAG-1 of claim 14, comprising the amino acid sequence shown in Figure 4 (SEQ ID NO: 4) or a peptide portion thereof.

16. The BAG-1 of claim 14, comprising the amino acid sequence shown in Figure 2 (SEQ ID NO: 2) or a peptide
25 portion thereof.

17. An antibody that specifically binds BAG-1 or a peptide portion thereof.

18. The antibody of claim 17, wherein said antibody is a monoclonal antibody.

19. A method for detecting the presence of a Bcl-2-related protein in a sample, comprising the steps of:

a. obtaining the sample;

5 b. adding to said sample a BAP under conditions that favor the binding of said BAP with the Bcl-2-related protein; and

c. detecting said bound Bcl-2-related protein.

10 20. The method of claim 19, wherein said BAP is BAG-1.

21. The method of claim 19, wherein said BAP is Raf-1.

15 22. A method of detecting an agent that can decrease or inhibit the binding of a BAP to a Bcl-2-related protein, comprising the steps of:

a. adding together in a first mixture the BAP and a Bcl-2-related protein under conditions that allow the binding of said BAP and said Bcl-2-related protein;

20 b. determining the amount of bound complex formed by the association of said BAP and said Bcl-2-related protein in said first mixture;

25 c. adding together in a second mixture the BAP, a Bcl-2-related protein and the agent under the conditions of step (a);

d. determining the amount of bound complex formed by the association of said BAP and said Bcl-2-related protein in said second mixture; and

5 e. detecting a decrease or inhibition of said binding due to said agent.

23. The method of claim 22, wherein said BAP is BAG-1.

24. The method of claim 22, wherein said BAP is Raf-1.

10 25. A method for detecting an agent that can induce the dissociation of a bound complex formed by the association of a BAP with a Bcl-2-related protein, comprising the steps of:

15 a. combining the BAP and the Bcl-2-related protein under conditions that favor association of said BAP to said Bcl-2-related protein;

b. determining the amount of bound complex formed by said association;

20 c. adding to said bound complex the agent; and

d. detecting the dissociation of said complex, wherein said dissociation indicates the presence of said dissociating agent.

25 26. The method of claim 25, wherein said BAP is BAG-1.

27. The method of claim 25, wherein said BAP is Raf-1.

28. A method for increasing the level of a BAP in a cell, comprising the steps of:

5 a. introducing into said cell a vector comprising the nucleic acid molecule of claim 10; and

 b. expressing said BAP or said active fragment thereof in said cell.

29. A method for decreasing the level of a BAP in a cell, comprising introducing into said cell an
10 antisense nucleotide sequence that is complementary to a portion of the nucleic acid molecule of claim 10, wherein said antisense nucleotide sequence specifically hybridizes to a nucleic acid sequence encoding the BAP in said cell.

30. The method of claim 29, wherein said nucleic
15 acid is DNA.

31. The method of claim 29, wherein said nucleic acid is mRNA.

32. A method for increasing the ability of a cell to survive, comprising the steps of:

20 a. introducing into said cell a vector comprising the nucleic acid molecule of claim 10; and

 b. expressing in said cell the BAP or active fragment thereof encoded by said nucleic
25 acid molecule.

33. The method of claim 32, further comprising the steps of:

5 c. introducing into said cell a vector comprising a nucleic acid sequence encoding a Bcl-2-related protein; and

d. expressing said Bcl-2-related protein in said cell.

34. The method of claim 33, wherein said Bcl-2-related protein is Bcl-2.

10 35. A method for decreasing the ability of a cell to survive, comprising introducing into said cell an antisense nucleotide sequence that is complementary to a region of the nucleic acid molecule of claim 10, wherein said antisense nucleotide sequence specifically hybridizes
15 to a nucleic acid sequence encoding a BAP in said cell.

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FIGURE 1

| 10 | 20 | 30 | 40 | 50 | |
|------------|-------------|-------------|-------------|-------------|------|
| 1234567890 | 1234567890 | 1234567890 | 1234567890 | 1234567890 | |
| CGGCTGCAAG | CGGCGGGTGA | AGAAGAAAGT | CGGGGGGGCG | TCTTCTCAGA | 50 |
| GCGAGAAGGT | AGGGAGGAGC | AGCAGGGAGT | TGACTAGTAG | TAAGAAAGTG | 100 |
| AACCGTAGCA | AGAACGTGAC | CGGGACCCAG | GTAGAGGAGG | TGACCAAGAT | 150 |
| CGAGGAGGGG | AACCAAAACG | AGGAAGTAAC | TGTGGCAGAA | GAGGTGACCC | 200 |
| AGACCGACAA | CATGGCCCAAG | AACGAGGAGA | TGGTCCAGAC | GGAGGAAATG | 250 |
| GAAACACCCA | GACTCAGCGT | CATGGTCACC | CACAGCAATG | AGAGGTATGA | 300 |
| CCCTCTTGTT | AACCCACAGC | AAGGTAACAG | TGAGCCAGTT | GTCCAAGACT | 350 |
| TGGCTCAGCT | TGTTCAAGAG | GCCACAGGAG | TTCACCTACC | TCTTCACAAG | 400 |
| CCTCATTTTA | AGGGAAAATC | TCCTGAAAGAA | ATGGAAACAC | CGTTGTCCAGC | 450 |
| ACCTTGAATG | CAAAATGGTT | GCCGAGTCAT | GTTAATTGGT | GAAAAGAGCA | 500 |
| ATCCAGAAGA | AGAGGTTGAG | TTAAGAAGC | TGAAAGATTT | GGAGGTATCT | 550 |
| GCAGAGAAGA | TAGCTAACCA | CCCTGCAAGAA | TTCGAATAAG | AGCTTTCCTGG | 600 |
| CATCCAGCAG | GGTTTCTCTGG | CTAAGGAATT | GCAAGCGGAG | GCTCTCTGCA | 650 |
| AACCTGATAG | GAAAGTAAAA | GCAACAATTG | AGCAATTTCAT | GAAGATCTTG | 700 |
| GAGGCGATTG | ACACAATGGT | CCCTACCAGAA | CAATTTTAAAG | ACAGCAGGCT | 750 |
| AAAAAGGAAG | AATTCTGGTGA | AAAAGGTTCA | GGTGTCTCTTA | GCAGAGTGTG | 800 |
| ACACAGTGGG | GCAATACATC | TGCCAAGAGA | CAGAGCGGCT | GCAGTCTACA | 850 |
| AACCTGGGCC | TGGCTCAATG | AAGTCCAGTG | GAGAGTGGCT | GTACTGGGCT | 900 |
| GAAGAGCAGC | TTTACAGGCC | TGCCCCCTCT | GGACAGGAAG | TGGCCTGTTT | 950 |
| CTCCATGGGT | GCCAGGGGCA | ACTAGGCCAA | TGTCAATTTT | CCCTGCTCTC | 1000 |
| CGTGGGTTCT | CAATGAAAAA | GTCCCTGCTT | TGCAACCTGA | AAAAAAA | 1050 |
| AAAA | | | | | 1054 |

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FIGURE 2

| 10 | 20 | 30 | 40 | 50 | |
|------------|------------|------------|-------------|------------|-----|
| 1234567890 | 1234567890 | 1234567890 | 1234567890 | 1234567890 | |
| MAKTEEMVQT | EEMETPRLSV | IVIHENERVD | LLVTPQOGNS | EPVVQDLAQL | 50 |
| VEEATGVPLP | FOKLIFRGKS | LKEMETPLSA | IGMONGCRVM | LIGESKNPEE | 100 |
| EVELKKLKDL | EVSAAKTANH | LQELNKELSG | IQQGFALAKEL | QAEALCKLDR | 150 |
| KVKATIEQFM | KILEEEDIMV | LPEQFKDSRL | KRKNLVKKVQ | VFLAECCTVE | 200 |
| QYICQETERL | QSTNLALAE | | | | 219 |

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FIGURE 3

| 10 | 20 | 30 | 40 | 50 | |
|-------------|------------|-------------|------------|------------|-----|
| 1234567890 | 1234567890 | 1234567890 | 1234567890 | 1234567890 | |
| CCCTTCATGTT | ACCTCCCAGC | AGGCCAGCAG | TGAACCAGTT | GTCCAAGACC | 50 |
| TGGCCCAGGT | TGTTGAAGAG | GTTCATAGGG | TTCCACAGTC | TTTTCAGAAA | 100 |
| CTCATATTTA | AGGGAAAATC | TCTGAAGGA | ATGGAAACAC | CGTTGTACAG | 150 |
| ACTTGGATA | CAAGATGGTT | GGCGGGTCAT | GTTAATTGGG | AAAAAGTACA | 200 |
| GTCCACAGGA | AGAGGTTGAA | CTAAAGAAGT | TGAAACATTT | GGAGAAGTCT | 250 |
| GTGGAGAAGA | TAGCTAACCA | GCTGGAAGAG | TTGAATAAAG | AGCTTACTGG | 300 |
| AATCCAGCAG | GGTTTTCTGC | CCAAGGATTT | GCAAGCTGAA | GCTCTCTGCA | 350 |
| AACITGATAG | GGAGTAAAA | GGCAATATAG | AGCAGTTTAT | GAAGATCTTG | 400 |
| GAGGAGATTG | ACACACIGAT | CCTGCCAGAA | AATTTCAAAG | ACAGTAGATT | 450 |
| GAAAAGGAAA | GGCTTGGTAA | AAAAGGTTCA | GGCATTCCTA | GGCGAGTGIG | 500 |
| ACACAGTGGG | GCAGAACATC | TGCCAGGAGA | CTGAGGGGCT | GCAGTCTACA | 550 |
| AACITTTGCCC | TGGCCGAGTG | AGGTTGTAGCA | GAAAAAGGCT | GTCCTGCCCT | 600 |
| GAAGAATGGC | GCCACCAGCT | CTGCCGTCTC | TGGATCGGAA | TTTACCTGAT | 650 |
| TTCTTCAGGG | CTGCTGGGGG | CAACTGGGCA | TTTGGCAATT | TTCTTACTCT | 700 |
| CACACTGGTT | CTCAATGAAA | AATAGTGTCT | TTG | | 733 |

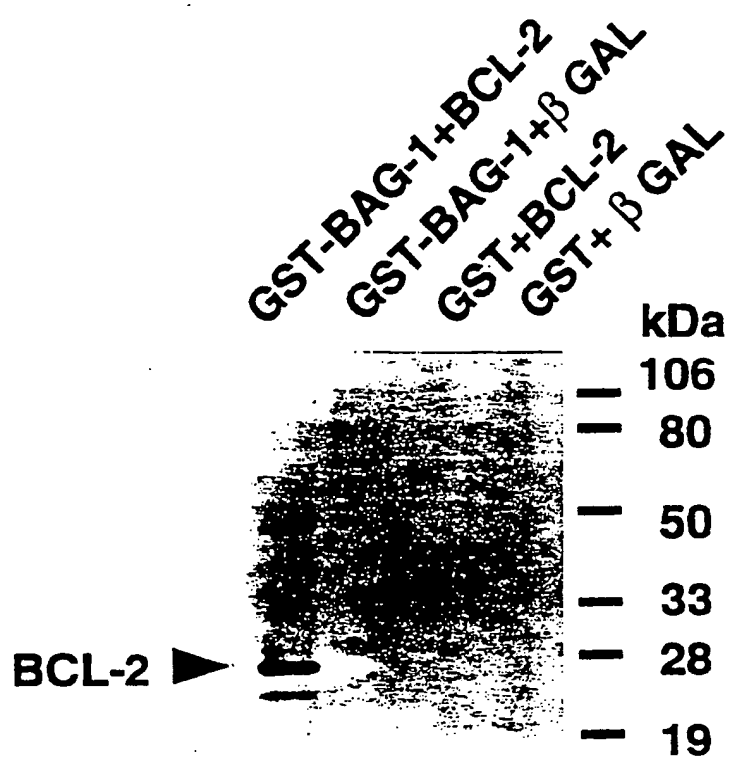
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FIGURE 4

| 10 | 20 | 30 | 40 | 50 | |
|------------|------------|------------|-------------|------------|-----|
| 1234567890 | 1234567890 | 1234567890 | 1234567890 | 1234567890 | |
| LHVTSQOGSS | EPVVOQLAQV | VEEVIGVPQS | FOKLIFKIGKS | LKEMETPLSA | 50 |
| LGIQDGCRVM | LIGKKNSPQE | EVELKKLKHL | EKSVEKIANQ | LEELNKELTG | 100 |
| IQOGFLPKDL | QAEALCKLDR | RVKATIEQFM | KILEETHILI | LPENFKDSRL | 150 |
| KRKGLVKKVQ | AFLAECCTVE | QNIQETERL | QSINFALAE | | 189 |

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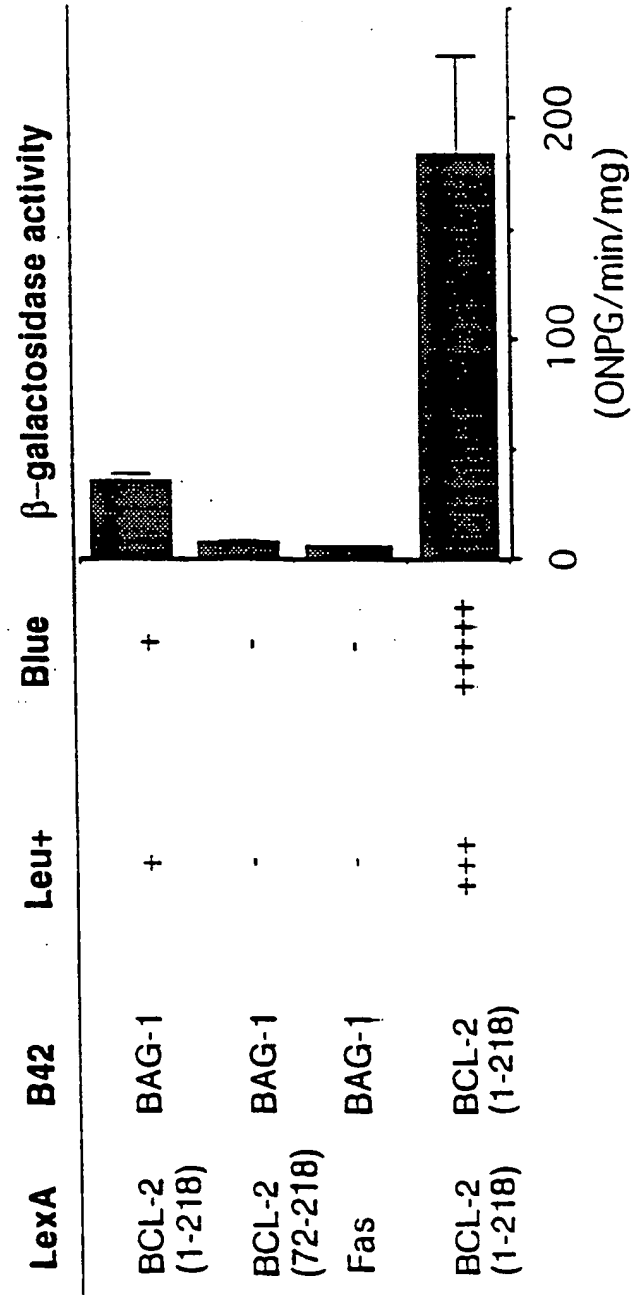
FIGURE 6



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FIGURE 7

YEAST TWO-HYBRID ANALYSIS OF BCL-2 AND BAG-1 INTERACTION



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FIGURE 8

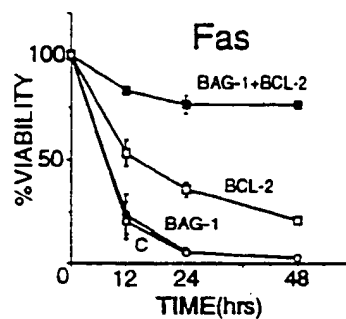


FIG. 8A

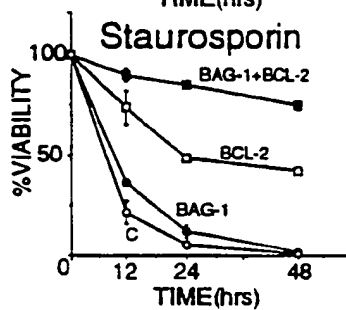


FIG. 8B

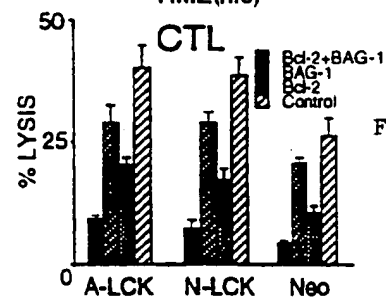


FIG. 8C

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FIGURE 9

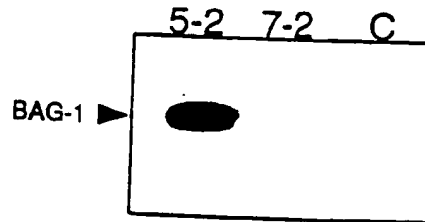


FIG. 9A

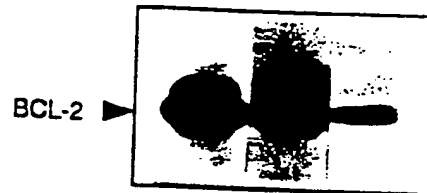


FIG. 9B

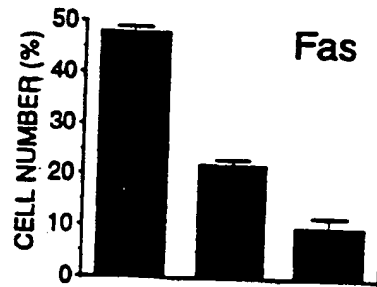


FIG. 9C

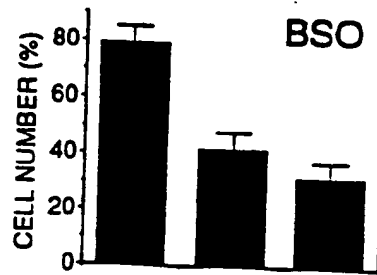


FIG. 9D

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FIGURE 10

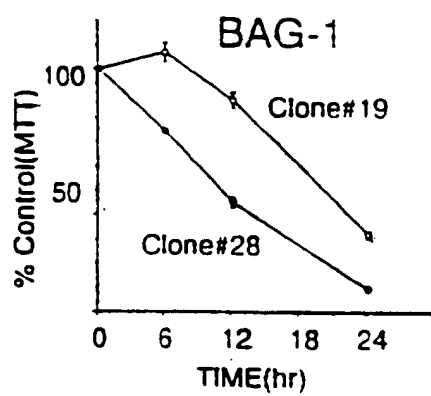


FIG. 10A

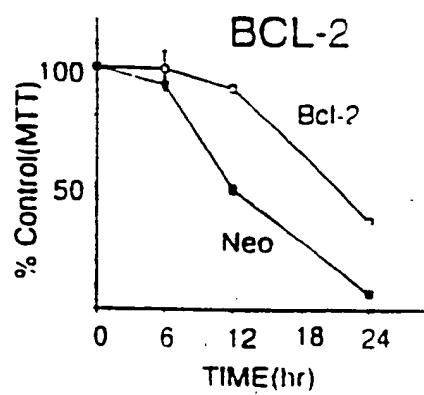
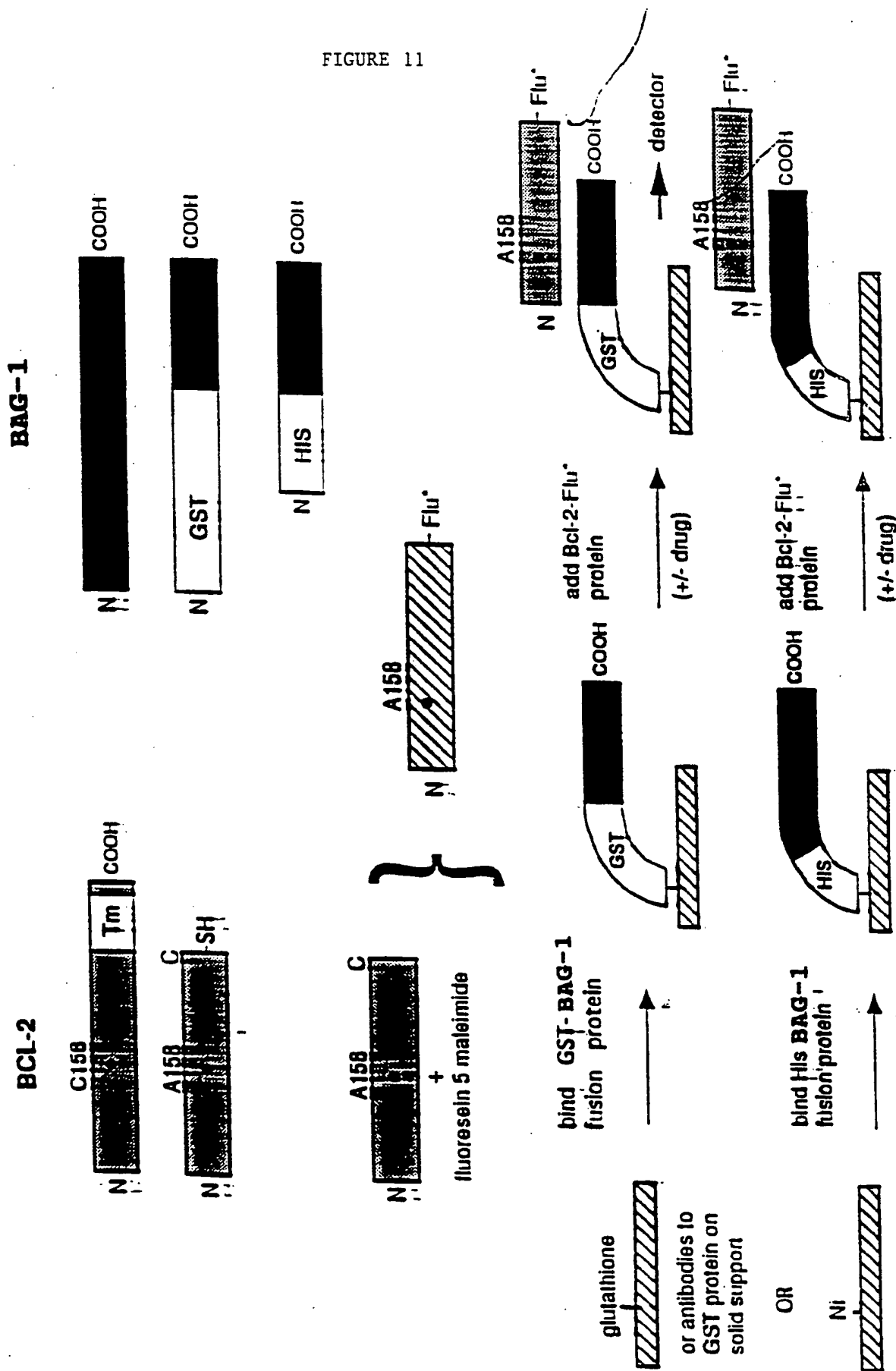


FIG. 10B

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DRUG SCREENING ASSAY FOR BLOCKERS OF BCL-2 INTERACTION WITH BAG-1



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12904

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 14/47; C12N 15/11, 15/63, 15/85; C07H 21/00

US CL : 536/23.5; 530/350; 435/69.1, 91.1, 320.1, 252.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 530/350; 435/69.1, 91.1, 320.1, 252.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Sequence search, APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| X | Cell, Volume 74, issued 27 August 1993, Z.N. Oltvai et al., "Bcl-2 Heterodimerizes In Vivo with a Conserved Homolog, Bax, That Accelerates Programmed Cell Death", pages 609-619, especially figure 2. | 1-3, 9-13, 19 |
| A | Science, Volume 258, issued 09 October 1992, I. Garcia et al., "Prevention of Programmed Cell Death of Sympathetic Neurons by the bcl-2 Proto-Oncogene", pages 302-304. | 1-35 |
| A | Nature, Volume 359, issued 08 October 1992, R.P. Bissonnette et al., "Apoptotic cell death induced by c-myc is inhibited by bcl-2", pages 552-554. | 1-35 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | |
|--|--|
| * Special categories of cited documents: | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" document defining the general state of the art which is not considered to be of particular relevance | "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" earlier document published on or after the international filing date | "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" document member of the same patent family |
| "O" document referring to an oral disclosure, use, exhibition or other means | |
| "P" document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

21 FEBRUARY 1995

Date of mailing of the international search report

13 MAR 1995

Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12904

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| A | Journal of Immunology, Volume 144, No. 9, issued 01 May 1990, G. Nunez et al., "Deregulated Bcl-2 Gene Expression Selectively Prolongs Survival of Growth Factor-Deprived Hemopoietic Cell Lines", pages 3602-3610. | 1-35 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12904

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12904

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-16, 19-35, drawn to a DNA encoding bag-1, the bag-1 protein, a method of detecting bag-1, and a method of cloning bag-1.

Group II, claims 17-18, drawn to an antibody to bag-1.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the antibody has a materially different structure and function as the DNA and the protein of group I.